DELIVERY OF OLIGONUCLEOTIDE COMPOUNDS INTO OSTEOCLASTS AND MODULATION OF OSTEOCLAST DIFFERENTIATION

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of United States patent application serial number 10/111,868, filed August 6, 2002, which is a national phase application of PCT/US00/29828, filed October 30, 2000, which claims priority to United States patent application serial number 09/435,296, filed November 5, 1999. The entire disclosures of these applications are hereby incorporated by reference in their entirety for all purposes.

BACKGROUND OF THE DISCLOSURE

FIELD OF THE DISCLOSURE

[0002] The present disclosure relates in general to methods of delivering oligonucleotide compounds into osteoclasts and osteoclast precursor cells. The present disclosure also relates to method of modulating osteoclast differentiation by introducing oligonucleotide compounds into osteoclast-like cells or osteoclast-precursor cells. Specifically, the present invention provides methods for delivering oligonucleotide compounds into osteoclasts and osteoclast precursor cells by transfecting the compounds into the target cells in the presence of a suitable non-liposomal transfection agent. The oligonucleotide compounds of the present disclosure are capable of binding a nucleic acid encoding RANK and modulating the expression of RANK. Upon transfection, these oligonucleotide compounds are capable of modulating differentiation of the osteoclast-like or osteoclast precursor cells into osteoclasts. The methods of the present disclosure may therefore be advantageously applied in the functional studies of osteoclasts and particularly the differentiation mechanisms of osteoclast-like cells. Modulation and

control of osteoclast differentiaion will be therapeutically useful to patients with subnormal bone conditions. The methods disclosed herein therefore may be utilized further in osteomedicine.

DESCRIPTION OF THE RELATED ART

[0003] Bone is permanently renewed by the coordinated actions of bone-resorbing osteoclasts and bone-forming osteoblasts, which metabolize and remodel bone structure during growth and adult life. Imbalances between osteoclast and osteoblast activities can result in skeletal abnormalities characterized by decreased (osteoporosis) or increase (osteopetrosis) bone mass. Both cell groups communicate with each other using cytokines and cell-cell contact to maintain bone homeostasis and these contacts are often mediated by the receptor RANK and its ligand RANKL. The production of RANKL is activated by T cells and can directly regulate osteoclastogenesis and bone remodeling, providing an explanation why autoimmune diseases, cancers, leukemia, asthma, and chronic viral infections such as hepatitis and HIV result in systemic and local bone loss. Several factors may contribute to the osteopenia that accompanies chronic illness, the most important being undernutrition and low body weight, inflammatory cytokines, disorders of the neuroendocrine axis (growth hormone/IGF-1 disturbances, thyroid and gonadal deficiency), immobilization, and the long-term use of glucocorticoids. The increased osteoclast activity is seen in many osteopenic disorders such as postmenopausal osteoporosis, Paget's disease, lytic bone metastases in breast or prostate cancers, or

increase bone resorption and crippling bone damage in arthritis (Kong and Penninger, *Exp Gerontol,* **2000**, *35*, 947-956.).

[0004] The origin of osteoblastic cells (osteoblasts, osteocytes, and bone-lining cells) differs from that of osteoclasts, with osteoblasts arising from mesenchymal stem cells while osteoclasts differentiate from hepatopoietic monocyte/macrophage precursors. As osteoblasts and osteoclasts derive from different cell types and perform different functions, their gene expression profiles are quite different. For example, osteoblasts express genes which participate in bone formation and mediate osteoclast activation, whereas osteoclast progenitor cells, following the initiation of osteoclastogenesis, express genes to permit cytoskeletal rearrangements, morphology changes and enzyme secretions required for bone degradation (Kong and Penninger, *Exp Gerontol,* **2000**, *35*, 947-956.). Oligonucleotides can be used to control the expression of bone cell-specific genes which regulate the differentiation and activity of bone cells. Delivery of such oligonucleotides into bone cells enables the regulation of cell differentiation and other functions of the bone.

[0005] Foreign nucleic acid molecules such as DNA molecules may be delivered into a cell by transfection. Transfection can be accomplished by mechanical and chemical methods. Non-viral gene transfer methods fall into two main categories: physical and chemical. The physical methods include, but are not limited to, electroporation (areas of cell membrane become porous through an electric pulse and DNA enters the cytoplasm), ballistic transfer (introduces particles coated with DNA into cells) and microinjection (DNA transfer through microcapillaries directly into cells).

Physical methods of gene transfer are limited by the low transfection efficiency of primary cells and high cell mortality. A commonly used method for chemical gene transfer achieved with the reagent Lipofectin. See, e.g., R.A. Olie et al., Cancer Research (60): 2805-2809. In this case, negatively charged DNA molecules bind to cationic lipids by electrostatic interaction and the DNA-lipid complex enters the cell through endocytosis. Another example of liposomal transfection agent is cytofectin. See, e.g., J.G. Lewis et al., Proc. Natl. Acad. Sci. USA (93):3176-3181.

[0006] There is a lack of success in transfecting osteoclasts with oligonucleotides reported in the art. The inability to deliver oligonucleotides into osteoclasts or osteoclast-like cells remains an experimental obstacle to the study of gene regulation and function of osteoclasts. It is known that osteoblasts and osteoblast-like cells can be transfected with either antisense oligonucleotides or plasmids using chemical methods. Antisense oligonucleotides have been transfected into several osteoblastic cell lines with lipid reagents, including the human periosteal osteoblast-like cells SaM-1 using the reagent FuGene (Ishibashi et al., *Biochim Biophys Acta,* **1999**, *1472*, 153-164.), and the mouse osteoblastic cells MC3T3-E1 using lipofectamine (Huang et al., *J Bone Miner Res,* **2000**, *15*, 188-197.; Takeshita et al., *J Biol Chem,* **1998**, *273*, 14738-14744.; You et al., *J Biol Chem,* **2002**, *277*, 48724-48729.). Plasmids have been transfected into several osteoblastic cell lines with lipid reagents, including: the mouse osteoblastic cells MC3T3-E1 using lipofectamine (Takeshita et al., *J Biol Chem,* **1998**, *273*, 14738-14744.), the human osteosarcoma cells MG-63 using the three reagents FuGene (Jones et al., *J Biol Chem,* **1999**, *274*, 32008-32014.), lipofectin (Riikonen et al., *J Biol Chem,* **1995**, *270*,

376-382.), and Tfx20 (Blair et al., *Biochem Biophys Res Commun,* **1999**, *255*, 778-784.), the human osteogenic sarcoma cells Saos2 using Tfx20 (Blair et al., *Biochem Biophys Res Commun,* **1999**, *255*, 778-784.), the human osteosarcoma cells HOS using lipofectin (Krueger et al., *Cancer Res,* **1999**, *59*, 6010-6014.; Riikonen et al., *J Biol Chem,* **1995**, *270*, 376-382.), and the rat osteoblast-like osteosarcoma cell line ROS using either lipofectin (Enomoto et al., *Biochem Biophys Res Commun,* **1993**, *191*, 1261-1269.), lipofectamine (Vander Molen et al., *J Biol Chem,* **1996**, *271*, 12165-12171.), or through an undisclosed method (Bowman et al., *J Bone Miner Res,* **1998**, *13*, 1700-1706.; Du et al., *Endocrine,* **2000**, *12*, 25-33.; Du et al., *Bone,* **2000**, *26*, 429-436.).

[0007] The above referenced reports describe transfection of oligonucleotides or plasmids into established cell lines. There have also been a few reports on transfection of primary cells, which are cells freshly isolated from a specific organ or tissue such as bone marrow or blood. Primary mouse osteoblasts have been transfected with antisense oligonucleotides using lipofectamine (Huang et al., *J Bone Miner Res,* **2000**, *15*, 188-197.), rat osteoblasts have been transfected with a plasmid using the reagent LT1 (McCarthy et al., *J Biol Chem,* **2000**, *275*, 21746-21753.), and rat bone marrow stromal cells which differentiated into osteoblasts were transfected with antisense oligonucleotides using ethoxylated polyethyleneimine (Gotoh et al., *Eur J Pharmacol,* **2002**, *451*, 19-25.). Kukita et al. reported that antisense oligonucleotides for an osteoclast-derived zinc finger protein (OCZF) inhibits the formation of osteoclast-like multinucleated cells in bone marrow culture. (Kukita et al., *Blood,* **1999**, *94*, 1987-1997.) The antisense oligonucleotides were added in that study to the bone marrow cell culture

to examine the effect on the cell differentiation. In the same studies, Kukita et al. also transfected OCZF cDNA into human kidney cells (293T cells) to investigate its role in transcriptional regulation.

[0008] The PCT publication WO 99/12567 discloses the addition of antisense oligonucleotides to co-cultures of osteoclast precursors and supporting cells (BLAIR et al., **1999**).

[0009] The US patent 5985554 discloses a method for probing the unknown function of a protein or peptide encoded by a cDNA, which comprises designing and synthesizing antisense oligonucleotide which is complementary to the sequence of the cDNA. This patent also discloses the construction of a system in which antisense oligonucleotides are added to an osteoclast culture system (Tanimura and Hosoya, **1999**).

[0010] The US pre-grant publication 20020106799 claims a method for transducing mammalian cells, including osteoclasts, with a retrovirally packaged foreign gene (Finer et al., **2002**).

[0011] In summary, no known reagents or methods have been reported to date that are capable of effectively transfecting osteoclasts with oligonucleotides. Liposomal transfection agents are considered to have detrimental effects on the primary bone marrow-derived osteoclasts cells when used to transfect the same. Nucleic acid molecule treatment alone has shown nonspecific effects and is ineffective. There remains a need for a method to deliver compounds such as oligonucleotides to developing bone or bone-derived precursor cells thereby regulating osteoclast differentiation and bone metabolism in general.

SUMMARY OF THE VARIOUS EMBODIMENTS

[0012] It is therefore an object of this disclosure to provide methods of delivering compounds, especially nucleic acid and nucleic acid-like oligomers, into osteoclasts or osteoclast precursor cells. In particular, methods are provided to deliver oligonucleotide compounds that target a nucleic acid encoding RANK and that are capable of modulating the expression of RANK into osteoclasts or osteoclast precursor cells. It is another object of this disclosure to provide methods for modulating osteoclast differentiation by delivering these compounds into osteoclast precursor cells. Delivery of these compounds may be carried out according to various embodiments by transfecting the compounds into osteoclasts, osteoclast-like cells, or osteoclast precursor cells in the presence of a non-liposomal transfection agent. Suitable non-liposomal transfection agents include, but are not limited to, Effectene® (Qiagen Inc.) and FuGENE 6 (Roche Diagnostics Corp.)

[0013] In accordance with the present disclosure, there is provided, in one embodiment, a method for delivering a compound 8 to 80 nucleobases in length into bone marrow derived osteoclast precursor cells. The method comprises transfecting the cells with the compound in the presence of a non-liposomal transfection agent. In one embodiment, the transfecting occurs during early differentiation of the bone marrow derived osteoclast precursor cells. In another embodiment, the bone marrow derived osteoclast precursor cells are cultured in the presence of RANK-ligand (RANKL) and macrophage colony stimulating factor (MCSF); the early differentiation is after day two

of culturing. In yet another embodiment, the early differentiation is before day four of the culturing.

[0014] In accordance with the present disclosure, there is provided, in another embodiment, a method for delivering a compound 8 to 80 nucleobases in length into a cell line whose cells are capable of differentiating into osteoclasts. The method comprises transfecting the cells of the cell line with the compound in the presence of a non-liposomal transfection agent. In another embodiment, the cell line is RAW264.7.

[0015] In accordance with the present disclosure, there is provided, in yet another embodiment, a method for delivering a compound 8 to 80 nucleobases in length into primary osteoclast cells. The method comprises transfecting the primary osteoclast cells with the compound in the presence of a non-liposomal transfection agent.

[0016] In accordance with the present disclosure, there is provided, in a further embodiment, a method for modulating osteoclast differentiation. The method comprises delivering a compound 8 to 80 nucleobases in length into bone marrow derived osteoclast precursor cells. The compound is targeted to a nucleic acid molecule encoding RANK and capable of binding a region of the nucleic acid molecule encoding RANK. The osteoclast differentiation of the bone marrow derived osteoclast precursor cells is modulated by the compound. In one embodiment, the delivery comprises transfecting the compound into the bone marrow derived osteoclast precursor cells. In another embodiment, the compound inhibits the expression of RANK mRNA by at least 10% upon transfection. In yet another embodiment, the transfecting is performed in the presence of a non-lipisomal transfection agent.

[0017] According to one embodiment, the non-lipisomal transfection agent is Effectene® or FuGENE 6.

[0018] According to another embodiment, the compound comprises 12 to 50 nucleobases in length. In yet another embodiment, the compound comprises 15 to 30 nucleobases in length. In still another embodiment, the compound comprises an oligonucleotide. In a further embodiment, the compound comprises an antisense oligonucleotide. In a still further embodiment, the compound comprises a DNA oligonucleotide. In another embodiment, the compound comprises RNA oligonucleotide. In yet another embodiment, the compound comprises a chimeric oligonucleotide. In still another embodiment, at least a portion of the compound hybridizes with RNA to form an oligonucleotide-RNA duplex.

[0019] According to a further embodiment, the compound is targeted to a nucleic acid molecule encoding RANK and capable of binding a region of the nucleic acid molecule encoding RANK. In one embodiment, the compound is at least 70% complementary to the region of the nucleic acid molecule encoding RANK. In another embodiment, the compound is at least 80% complementary to the region of the nucleic acid molecule encoding RANK. In yet another embodiment, the compound is at least 90% complementary to the region of the nucleic acid molecule encoding RANK. In still another embodiment, the compound is at least 95% complementary to the region of the nucleic acid molecule encoding RANK. In a further embodiment, the compound is at least 99% complementary to the region of the nucleic acid molecule encoding RANK.

DETAILED DESCRIPTION OF THE VARIOUS EMBODIMENTS

Brief Discussion of Relevant Terms

[0020] Compounds, particularly oligonucleotides and similar species, may be used in modulating the function or effect of nucleic acid molecules encoding RANK. The present disclosure provides oligonucleotides that specifically hybridize with one or more nucleic acid molecules encoding RANK. As used herein, the terms "target nucleic acid" and "nucleic acid molecule encoding RANK" have been used for convenience to encompass DNA encoding RANK, RNA (including pre-mRNA and mRNA or portions thereof) transcribed from such DNA, and also cDNA derived from such RNA. A compound of this invention that hybridizes with its target nucleic acid is generally referred to as an "antisense compound." Consequently, the preferred mechanism believed to be included in the practice of some preferred embodiments of the invention is referred to herein as "antisense inhibition." Such antisense inhibition is typically based upon hydrogen bonding-based hybridization of oligonucleotide strands or segments such that at least one strand or segment is cleaved, degraded, or otherwise rendered inoperable. In this regard, it is presently preferred to target specific nucleic acid molecules and their functions for such antisense inhibition.

[0021] The functions of DNA to be interfered with can include replication and transcription. Replication and transcription, for example, can be from an endogenous cellular template, a vector, a plasmid construct or otherwise. The functions of RNA to be

interfered with can include functions such as translocation of the RNA to a site of protein translation, translocation of the RNA to sites within the cell which are distant from the site of RNA synthesis, translation of protein from the RNA, splicing of the RNA to yield one or more RNA species, and catalytic activity or complex formation involving the RNA which may be engaged in or facilitated by the RNA. One preferred result of such interference with target nucleic acid function is modulation of the expression of RANK. In the context of the present invention, "modulation" and "modulation of expression" mean either an increase (stimulation) or a decrease (inhibition) in the amount or levels of a nucleic acid molecule encoding the gene, e.g., DNA or RNA. Inhibition is often the preferred form of modulation of expression and mRNA is often a preferred target nucleic acid.

[0022] In the context of this invention, "hybridization" means the pairing of complementary strands of oligomeric compounds. In the present invention, the preferred mechanism of pairing involves hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases (nucleobases) of the strands of oligomeric compounds. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. Hybridization can occur under varying circumstances.

[0023] An antisense compound is specifically hybridizable when binding of the compound to the target nucleic acid interferes with the normal function of the target nucleic acid to cause a loss of activity, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target nucleic acid

sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and under conditions in which assays are performed in the case of *in vitro* assays.

[0024] In the present invention the phrase "stringent hybridization conditions" or "stringent conditions" refers to conditions under which a compound of the invention will hybridize to its target sequence, but to a minimal number of other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances and in the context of this invention, "stringent conditions" under which oligomeric compounds hybridize to a target sequence are determined by the nature and composition of the oligomeric compounds and the assays in which they are being investigated.

[0025] "Complementary," as used herein, refers to the capacity for precise pairing between two nucleobases of an oligomeric compound. For example, if a nucleobase at a certain position of an oligonucleotide (an oligomeric compound), is capable of hydrogen bonding with a nucleobase at a certain position of a target nucleic acid, said target nucleic acid being a DNA, RNA, or oligonucleotide molecule, then the position of hydrogen bonding between the oligonucleotide and the target nucleic acid is considered to be a complementary position. The oligonucleotide and the further DNA, RNA, or oligonucleotide molecule are complementary to each other when a sufficient number of complementary positions in each molecule are occupied by nucleobases which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of precise pairing or

complementarity over a sufficient number of nucleobases such that stable and specific binding occurs between the oligonucleotide and a target nucleic acid.

[0026] It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. Moreover, an oligonucleotide may hybridize over one or more segments such that intervening or adjacent segments are not involved in the hybridization event (e.g., a loop structure or hairpin structure). It is preferred that the antisense compounds of the present invention comprise at least 70%, or at least 75%, or at least 80%, or at least 85% sequence complementarity to a target region within the target nucleic acid, more preferably that they comprise at least 90% sequence complementarity and even more preferably comprise at least 95% or at least 99% sequence complementarity to the target region within the target nucleic acid sequence to which they are targeted. For example, an antisense compound in which 18 of 20 nucleobases of the antisense compound are complementary to a target region, and would therefore specifically hybridize, would represent 90 percent complementarity. In this example, the remaining noncomplementary nucleobases may be clustered or interspersed with complementary nucleobases and need not be contiguous to each other or to complementary nucleobases. As such, an antisense compound which is 18 nucleobases in length having 4 (four) noncomplementary nucleobases which are flanked by two regions of complete complementarity with the target nucleic acid would have 77.8% overall complementarity with the target nucleic acid and would thus fall within the scope of the present invention. Percent complementarity of an antisense compound with a region of a target nucleic acid can be

determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the art (Altschul et al., *J. Mol. Biol.*, **1990**, 215, 403-410; Zhang and Madden, *Genome Res.*, **1997**, 7, 649-656).

[0027] Percent homology, sequence identity or complementarity, can be determined by, for example, the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison WI), using default settings, which uses the algorithm of Smith and Waterman (Adv. Appl. Math., 1981, 2, 482-489). In some preferred embodiments, homology, sequence identity or complementarity, between the oligomeric and target is between about 50% to about 60%. In some embodiments, homology, sequence identity or complementarity, is between about 60% to about 70%. In preferred embodiments, homology, sequence identity or complementarity, is between about 70% and about 80%. In more preferred embodiments, homology, sequence identity or complementarity, is between about 80% and about 90%. In some preferred embodiments, homology, sequence identity or complementarity, is about 90%, about 92%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99%.

Compounds

[0028] According to the present invention, compounds include antisense oligomeric compounds, antisense oligonucleotides, ribozymes, external guide sequence (EGS) oligonucleotides, alternate splicers, primers, probes, and other oligomeric compounds which hybridize to at least a portion of the target nucleic acid. As such, these compounds

may be introduced in the form of single-stranded, double-stranded, circular or hairpin oligomeric compounds and may contain structural elements such as internal or terminal bulges or loops. Once introduced to a system, the compounds of the invention may elicit the action of one or more enzymes or structural proteins to effect modification of the target nucleic acid.

[0029] One non-limiting example of such an enzyme is RNAse H, a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. It is known in the art that single-stranded antisense compounds which are "DNA-like" elicit RNAse H. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide-mediated inhibition of gene expression. Similar roles have been postulated for other ribonucleases such as those in the RNase III and ribonuclease L family of enzymes.

[0030] While the preferred form of antisense compound is a single-stranded antisense oligonucleotide, in many species the introduction of double-stranded structures, such as double-stranded RNA (dsRNA) molecules, has been shown to induce potent and specific antisense-mediated reduction of the function of a gene or its associated gene products. This phenomenon occurs in both plants and animals and is believed to have an evolutionary connection to viral defense and transposon silencing. Accordingly, in one embodiment of the invention, the antisense compound is a double stranded structure, e.g., a dsRNA.

[0031] The first evidence that dsRNA could lead to gene silencing in animals came in 1995 from work in the nematode, *Caenorhabditis elegans* (Guo and Kempheus, *Cell*,

1995, 81, 611-620). Montgomery et al. have shown that the primary interference effects of dsRNA are posttranscriptional (Montgomery et al., *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 15502-15507). The posttranscriptional antisense mechanism defined in *Caenorhabditis elegans* resulting from exposure to double-stranded RNA (dsRNA) has since been designated RNA interference (RNAi). This term has been generalized to mean antisense-mediated gene silencing involving the introduction of dsRNA leading to the sequence-specific reduction of endogenous targeted mRNA levels (Fire et al., *Nature*, **1998**, 391, 806-811). Recently, it has been shown that it is, in fact, the single-stranded RNA oligomers of antisense polarity of the dsRNAs which are the potent inducers of RNAi (Tijsterman et al., *Science*, **2002**, *295*, 694-697).

[0032] The oligonucleotides of the present invention also include modified oligonucleotides in which a different base is present at one or more of the nucleotide positions in the oligonucleotide, as long as the structural and functional elements are maintained, e.g., the modified oligonucleotide hybridizes to and inhibits the expression of the target gene. For example, if the first nucleotide is an adenosine, modified oligonucleotides may be produced which contain thymidine, guanosine or cytidine at this position. This may be done at any of the positions of the oligonucleotide. These oligonucleotides are then tested using the methods described herein to determine their ability to inhibit expression of RANK mRNA.

[0033] In the context of this invention, the term "oligomeric compound" refers to a polymer or oligomer comprising a plurality of monomeric units. In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid

(RNA) or deoxyribonucleic acid (DNA) or mimetics, chimeras, analogs and homologs thereof. This term includes oligonucleotides composed of naturally occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for a target nucleic acid and increased stability in the presence of nucleases.

[0034] While oligonucleotides are a preferred form of the compounds of this invention, the present invention comprehends other families of compounds as well, including but not limited to oligonucleotide analogs and mimetics such as those described herein.

[0035] The compounds in accordance with this invention preferably comprise from about 8 to about 80 nucleobases (i.e. from about 8 to about 80 linked nucleosides). One of ordinary skill in the art will appreciate that the invention embodies compounds of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, or 80 nucleobases in length.

[0036] In one preferred embodiment, the compounds of the invention are 12 to 50 nucleobases in length. One having ordinary skill in the art will appreciate that this embodies compounds of 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28,

29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleobases in length.

[0037] In another preferred embodiment, the compounds of the invention are 15 to 30 nucleobases in length. One having ordinary skill in the art will appreciate that this embodies compounds of 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleobases in length.

[0038] Particularly preferred compounds are oligonucleotides from about 12 to about 50 nucleobases, even more preferably those comprising from about 15 to about 30 nucleobases.

[0039] Antisense compounds 8-80 nucleobases in length comprising a stretch of at least eight (8) consecutive nucleobases selected from within the illustrative antisense compounds are considered to be suitable antisense compounds as well.

[0040] Exemplary preferred antisense compounds include oligonucleotide sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative preferred antisense compounds (the remaining nucleobases being a consecutive stretch of the same oligonucleotide beginning immediately upstream of the 5'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the oligonucleotide contains about 8 to about 80 nucleobases). Similarly preferred antisense compounds are represented by oligonucleotide sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative preferred antisense compounds (the remaining nucleobases being a consecutive stretch of the same oligonucleotide beginning

immediately downstream of the 3'-terminus of the antisense compound which is specifically hybridizable to the target nucleic acid and continuing until the oligonucleotide contains about 8 to about 80 nucleobases). One having skill in the art armed with the preferred antisense compounds illustrated herein will be able, without undue experimentation, to identify further preferred antisense compounds.

Targeting a Nucleic Acid Molecule

[0041] "Targeting" an antisense compound to a particular nucleic acid molecule, in the context of this invention, can be a multistep process. The process usually begins with the identification of a target nucleic acid whose function is to be modulated. This target nucleic acid may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target nucleic acid encodes RANK.

[0042] The targeting process usually also includes determination of at least one target region, segment, or site within the target nucleic acid for the antisense interaction to occur such that the desired effect, e.g., modulation of expression, will result. Within the context of the present invention, the term "region" is defined as a portion of the target nucleic acid having at least one identifiable structure, function, or characteristic. Within regions of target nucleic acids are segments. "Segments" are defined as smaller or sub-portions of regions within a target nucleic acid. "Sites," as used in the present invention, are defined as positions within a target nucleic acid.

[0043] Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA transcribed from a gene encoding RANK, regardless of the sequence(s) of such codons. It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively).

[0044] The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to

about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon. Consequently, the "start codon region" (or "translation initiation codon region") and the "stop codon region" (or "translation termination codon region") are all regions which may be targeted effectively with the antisense compounds of the present invention.

[0045] The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Within the context of the present invention, a preferred region is the intragenic region encompassing the translation initiation or termination codon of the open reading frame (ORF) of a gene.

[0046] Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA (or corresponding nucleotides on the gene), and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA (or corresponding nucleotides on the gene). The 5' cap site of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap site. It is also preferred to target the 5' cap region.

[0047] Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. Targeting splice sites, i.e., intron-exon junctions or exon-intron junctions, may also be particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred target sites. mRNA transcripts produced via the process of splicing of two (or more) mRNAs from different gene sources are known as "fusion transcripts". It is also known that introns can be effectively targeted using antisense compounds targeted to, for example, DNA or pre-mRNA.

[0048] It is also known in the art that alternative RNA transcripts can be produced from the same genomic region of DNA. These alternative transcripts are generally known as "variants". More specifically, "pre-mRNA variants" are transcripts produced from the same genomic DNA that differ from other transcripts produced from the same genomic DNA in either their start or stop position and contain both intronic and exonic sequence.

[0049] Upon excision of one or more exon or intron regions, or portions thereof during splicing, pre-mRNA variants produce smaller "mRNA variants". Consequently, mRNA variants are processed pre-mRNA variants and each unique pre-mRNA variant must always produce a unique mRNA variant as a result of splicing. These mRNA

variants are also known as "alternative splice variants". If no splicing of the pre-mRNA variant occurs then the pre-mRNA variant is identical to the mRNA variant.

[0050] It is also known in the art that variants can be produced through the use of alternative signals to start or stop transcription and that pre-mRNAs and mRNAs can possess more that one start codon or stop codon. Variants that originate from a pre-mRNA or mRNA that use alternative start codons are known as "alternative start variants" of that pre-mRNA or mRNA. Those transcripts that use an alternative stop codon are known as "alternative stop variants" of that pre-mRNA or mRNA. One specific type of alternative stop variant is the "polyA variant" in which the multiple transcripts produced result from the alternative selection of one of the "polyA stop signals" by the transcription machinery, thereby producing transcripts that terminate at unique polyA sites. Within the context of the invention, the types of variants described herein are also preferred target nucleic acids.

[0051] The locations on the target nucleic acid to which the preferred antisense compounds hybridize are hereinbelow referred to as "preferred target segments." As used herein the term "preferred target segment" is defined as at least an 8-nucleobase portion of a target region to which an active antisense compound is targeted. While not wishing to be bound by theory, it is presently believed that these target segments represent portions of the target nucleic acid which are accessible for hybridization.

[0052] While the specific sequences of certain preferred target segments are set forth herein, one of skill in the art will recognize that these serve to illustrate and describe

particular embodiments within the scope of the present invention. Additional preferred target segments may be identified by one having ordinary skill.

[0053] Target segments 8-80 nucleobases in length comprising a stretch of at least eight (8) consecutive nucleobases selected from within the illustrative preferred target segments are considered to be suitable for targeting as well.

[0054] Target segments can include DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative preferred target segments (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately upstream of the 5'-terminus of the target segment and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). Similarly preferred target segments are represented by DNA or RNA sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative preferred target segments (the remaining nucleobases being a consecutive stretch of the same DNA or RNA beginning immediately downstream of the 3'-terminus of the target segment and continuing until the DNA or RNA contains about 8 to about 80 nucleobases). One having skill in the art armed with the preferred target segments illustrated herein will be able, without undue experimentation, to identify further preferred target segments.

[0055] Once one or more target regions, segments or sites have been identified, antisense compounds are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

[0056] The oligomeric compounds are also targeted to or not targeted to regions of the target nucleobase sequence (e.g., such as those disclosed in Example 13) comprising nucleobases 1-50, 51-100, 101-150, 151-200, 201-250, 251-300, 301-350, 351-400, 401-450, 451-500, 501-550, 551-600, 601-650, 651-700, 701-750, 751-800, 801-850, 851-900, 901-950, 951-1000, 1001-1050, 1051-1100, 1101-1150, 1151-1200, 1201-1250, 1251-1300, 1301-1350, 1351-1400, 1401-1450, 1451-1500, 1501-1550, 1551-1600, 1601-1650, 1651-1700, 1701-1750, 1751-1800, 1801-1850, 1851-1900, 1901-1950, 1951-2000, 2001-2050, 2051-2100, 2101-2116, or any combination thereof.

Screening and Target Validation

[0057] In a further embodiment, the "preferred target segments" identified herein may be employed in a screen for additional compounds that modulate the expression of RANK. "Modulators" are those compounds that decrease or increase the expression of a nucleic acid molecule encoding RANK and which comprise at least an 8-nucleobase portion which is complementary to a preferred target segment. The screening method comprises the steps of contacting a preferred target segment of a nucleic acid molecule encoding RANK with one or more candidate modulators, and selecting for one or more candidate modulators which decrease or increase the expression of a nucleic acid molecule encoding RANK. Once it is shown that the candidate modulator or modulators are capable of modulating (e.g. either decreasing or increasing) the expression of a nucleic acid molecule encoding RANK, the modulator may then be employed in further

investigative studies of the function of RANK, or for use as a research, diagnostic, or therapeutic agent in accordance with the present invention.

[0058] The preferred target segments of the present invention may be also be combined with their respective complementary antisense compounds of the present invention to form stabilized double-stranded (duplexed) oligonucleotides.

[0059] Such double stranded oligonucleotide moieties have been shown in the art to modulate target expression and regulate translation as well as RNA processsing via an antisense mechanism. Moreover, the double-stranded moieties may be subject to chemical modifications (Fire et al., *Nature*, **1998**, *391*, 806-811; Timmons and Fire, *Nature* **1998**, *395*, 854; Timmons et al., *Gene*, **2001**, *263*, 103-112; Tabara et al., *Science,* **1998**, 282, 430-431; Montgomery et al., *Proc. Natl. Acad. Sci. USA*, **1998**, *95*, 15502-15507; Tuschl et al., *Genes Dev.*, **1999**, *13*, 3191-3197; Elbashir et al., *Nature*, **2001**, *411*, 494-498; Elbashir et al., *Genes Dev.* **2001**, *15*, 188-200). For example, such double-stranded moieties have been shown to inhibit the target by the classical hybridization of antisense strand of the duplex to the target, thereby triggering enzymatic degradation of the target (Tijsterman et al., *Science*, **2002**, *295*, 694-697).

[0060] The compounds of the present invention can also be applied in the areas of drug discovery and target validation. The present invention comprehends the use of the compounds and preferred target segments identified herein in drug discovery efforts to elucidate relationships that exist between RANK and a disease state, phenotype, or condition. These methods include detecting or modulating RANK comprising contacting a sample, tissue, cell, or organism with the compounds of the present invention,

measuring the nucleic acid or protein level of RANK and/or a related phenotypic or chemical endpoint at some time after treatment, and optionally comparing the measured value to a non-treated sample or sample treated with a further compound of the invention. These methods can also be performed in parallel or in combination with other experiments to determine the function of unknown genes for the process of target validation or to determine the validity of a particular gene product as a target for treatment or prevention of a particular disease, condition, or phenotype.

Kits, Research Reagents, Diagnostics, and Therapeutics

[0061] The compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. Furthermore, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes or to distinguish between functions of various members of a biological pathway.

[0062] For use in kits and diagnostics, the compounds of the present invention, either alone or in combination with other compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues.

[0063] As one nonlimiting example, expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential

levels of gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds which affect expression patterns.

[0064] Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, **2000,** 480, 17-24; Celis, *et al.*, *FEBS Lett.*, **2000,** 480, 2-16), SAGE (serial analysis of gene expression)(Madden, *et al.*, *Drug Discov. Today,* **2000,** 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, **1999,** 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, *et al.*, *Proc. Natl. Acad. Sci. U. S. A.*, **2000,** 97, 1976-81), protein arrays and proteomics (Celis, *et al.*, *FEBS Lett.*, **2000,** 480, 2-16; Jungblut, *et al.*, *Electrophoresis,* **1999,** 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, *et al.*, *FEBS Lett.*, **2000,** 480, 2-16; Larsson, *et al.*, *J. Biotechnol.*, **2000,** 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, *et al.*, *Anal. Biochem.*, **2000,** 286, 91-98; Larson, *et al.*, *Cytometry,* **2000,** 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*, **2000,** 3, 316-21), comparative genomic hybridization (Carulli, *et al.*, *J. Cell Biochem. Suppl.*, **1998,** 31, 286-96), FISH (fluorescent *in situ* hybridization) techniques (Going and Gusterson, *Eur. J. Cancer,* **1999,** 35, 1895-904) and mass spectrometry methods (To, *Comb. Chem. High Throughput Screen,* **2000,** 3, 235-41).

[0065] The compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding RANK. For example,

oligonucleotides that are shown to hybridize with such efficiency and under such conditions as disclosed herein as to be effective RANK inhibitors will also be effective primers or probes under conditions favoring gene amplification or detection, respectively. These primers and probes are useful in methods requiring the specific detection of nucleic acid molecules encoding RANK and in the amplification of said nucleic acid molecules for detection or for use in further studies of RANK. Hybridization of the antisense oligonucleotides, particularly the primers and probes, of the invention with a nucleic acid encoding RANK can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of RANK in a sample may also be prepared.

[0066] The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense compounds have been employed as therapeutic moieties in the treatment of disease states in animals, including mouses. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to mouses and numerous clinical trials are presently underway. It is thus established that antisense compounds can be useful therapeutic modalities that can be configured to be useful in treatment regimes for the treatment of cells, tissues and animals, especially mouses.

[0067] For therapeutics, an animal, preferably a mouse, suspected of having a disease or disorder which can be treated by modulating the expression of RANK is treated by administering antisense compounds in accordance with this invention. For

example, in one non-limiting embodiment, the methods comprise the step of administering to the animal in need of treatment, a therapeutically effective amount of a RANK inhibitor. The RANK inhibitors of the present invention effectively inhibit the activity of the RANK protein or inhibit the expression of the RANK protein. In one embodiment, the activity or expression of RANK in an animal is inhibited by about 10%. Preferably, the activity or expression of RANK in an animal is inhibited by about 30%. More preferably, the activity or expression of RANK in an animal is inhibited by 50% or more. Thus, the oligomeric compounds modulate expression of RANK mRNA by at least 10%, by at least 20%, by at least 25%, by at least 30%, by at least 40%, by at least 50%, by at least 60%, by at least 70%, by at least 75%, by at least 80%, by at least 85%, by at least 90%, by at least 95%, by at least 98%, by at least 99%, or by 100%.

[0068] For example, the reduction of the expression of RANK may be measured in serum, adipose tissue, liver or any other body fluid, tissue or organ of the animal. Preferably, the cells contained within said fluids, tissues or organs being analyzed contain a nucleic acid molecule encoding RANK protein and/or the RANK protein itself.

[0069] The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of a compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the compounds and methods of the invention may also be useful prophylactically.

Modifications

[0070] As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn, the respective ends of this linear polymeric compound can be further joined to form a circular compound, however, linear compounds are generally preferred. In addition, linear compounds may have internal nucleobase complementarity and may therefore fold in a manner as to produce a fully or partially double-stranded compound. Within oligonucleotides, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Modified Internucleoside Linkages (Backbones)

[0071] Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a

phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

[0072] Preferred modified oligonucleotide backbones containing a phosphorus atom therein include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

[0073] Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are

commonly owned with this application, and each of which is herein incorporated by reference.

[0074] Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

[0075] Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Modified Sugar and Internucleoside linkages-Mimetics

[0076] In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage (i.e. the backbone), of the nucleotide units are replaced with novel groups. The nucleobase units are maintained for hybridization with an appropriate target nucleic acid. One such compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen *et al.*, *Science*, **1991**, 254, 1497-1500.

[0077] Preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known as a methylene (methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ [wherein the native phosphodiester backbone is represented as $-O-P-O-CH_2-$] of the above referenced U.S. patent 5,489,677, and the amide backbones

of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified Sugars

[0078] Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, **1995**, *78*, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as

described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethyl-amino-ethoxy-ethyl or 2'-DMAEOE), i.e., 2'-O-CH_2-O-CH_2-$N(CH_3)_2$, also described in examples hereinbelow.

[0079] Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-allyl (2'-CH_2-CH=CH_2), 2'-O-allyl (2'-O-CH_2-CH=CH_2) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

[0080] A further preferred modification of the sugar includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring, thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene (-CH_2-)$_n$ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Natural and Modified Nucleobases

[0081] Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-CH_3) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine(1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified

nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those disclosed by Englisch *et al.*, *Angewandte Chemie*, International Edition, **1991**, *30*, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302; Crooke, S.T. and Lebleu, B. , ed., CRC Press, **1993**. Certain of these nucleobases are particularly useful for increasing the binding affinity of the compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyl-adenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 °C and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

[0082] Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United

States patent 5,750,692, which is commonly owned with the instant application and also herein incorporated by reference.

Conjugates

[0083] Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. These moieties or conjugates can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugate groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve uptake, enhance resistance to degradation, and/or strengthen sequence-specific hybridization with the target nucleic acid. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve uptake, distribution, metabolism or excretion of the compounds of the present invention. Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992, and U.S. Patent 6,287,860, the entire disclosure of which are incorporated herein by reference. Conjugate moieties include but

are not limited to lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g., hexyl-S-tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety. Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fenbufen, ketoprofen, (*S*)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

[0084] Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928

and 5,688,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

Chimeric Compounds

[0085] It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide.

[0086] The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, increased stability and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNAse H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide-mediated inhibition of gene expression. The cleavage of RNA:RNA hybrids can, in like fashion, be accomplished through the actions

of endoribonucleases, such as RNAseL which cleaves both cellular and viral RNA. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

[0087] Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Formulations

[0088] The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor-targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption-assisting formulations include, but are not limited to, U.S.: 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221;

5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

[0089] The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal, including a mouse, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

[0090] The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin *et al.*, published December 9, 1993 or in WO 94/26764 and U.S. 5,770,713 to Imbach *et al.*

[0091] The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. For oligonucleotides, preferred examples of

pharmaceutically acceptable salts and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

[0092] The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration. Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

[0093] The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the

pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

[0094] The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

[0095] Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, foams and liposome-containing formulations. The pharmaceutical compositions and formulations of the present invention may comprise one or more penetration enhancers, carriers, excipients or other active or inactive ingredients.

[0096] Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. Emulsions may contain additional components in addition to the dispersed phases, and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Microemulsions are included as an embodiment of the present invention.

Emulsions and their uses are well known in the art and are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

[0097] Formulations of the present invention include liposomal formulations. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers. Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior that contains the composition to be delivered. Cationic liposomes are positively charged liposomes which are believed to interact with negatively charged DNA molecules to form a stable complex. Liposomes that are pH-sensitive or negatively-charged are believed to entrap DNA rather than complex with it. Both cationic and noncationic liposomes have been used to deliver DNA to cells.

[0098] Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome comprises one or more glycolipids or is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. Liposomes and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

[0099] The pharmaceutical formulations and compositions of the present invention may also include surfactants. The use of surfactants in drug products, formulations and in

emulsions is well known in the art. Surfactants and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

[00100] In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly oligonucleotides. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs. Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants. Penetration enhancers and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety.

[00101] One of skill in the art will recognize that formulations are routinely designed according to their intended use, i.e. route of administration.

[0100] Preferred formulations for topical administration include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA).

[0101] For topical or other administration, oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic

liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids. Preferred fatty acids and esters, pharmaceutically acceptable salts thereof, and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999, which is incorporated herein by reference in its entirety.

[0102] Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts and fatty acids and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its entirety. Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents and their uses are further described in U.S. Patent 6,287,860, which is incorporated herein in its

entirety. Oral formulations for oligonucleotides and their preparation are described in detail in United States applications 09/108,673 (filed July 1, 1998), 09/315,298 (filed May 20, 1999) and 10/071,822, filed February 8, 2002, each of which is incorporated herein by reference in their entirety.

[0103] Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

[0104] Certain embodiments of the invention provide pharmaceutical compositions containing one or more oligomeric compounds and one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include but are not limited to cancer chemotherapeutic drugs such as daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16), trimetrexate, irinotecan, topotecan, gemcitabine, teni-

poside, cisplatin and diethylstilbestrol (DES). When used with the compounds of the invention, such chemotherapeutic agents may be used individually (*e.g.*, 5-FU and oligonucleotide), sequentially (*e.g.*, 5-FU and oligonucleotide for a period of time followed by MTX and oligonucleotide), or in combination with one or more other such chemotherapeutic agents (*e.g.*, 5-FU, MTX and oligonucleotide, or 5-FU, radiotherapy and oligonucleotide). Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and ganciclovir, may also be combined in compositions of the invention. Combinations of antisense compounds and other non-antisense drugs are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

[0105] In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Alternatively, compositions of the invention may contain two or more antisense compounds targeted to different regions of the same nucleic acid target. Numerous examples of antisense compounds are known in the art. Two or more combined compounds may be used together or sequentially.

Dosing

[0106] The formulation of therapeutic compositions and their subsequent administration (dosing) is believed to be within the skill of those in the art. Dosing is

dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

Transfection and Cellular Delivery of Oligonucleotide Compounds

[0107] Oligonucleotide compounds may be delivered into a cell by way of transfection. Procedures and methodologies of transfection are generally known to an ordinarily skilled molecular biologist. See, in general, Sambrook et al., 2001 Molecular

Cloning: A Laboratory Manual, 3rd ed. vol. 1-3, Cold Spring Harbor Press. See also, the discussion supra in the Description of the Related Art. Various transfection agents have been developed that enhance the cellular uptake of oligonucleotides, particularly the uptake of antisense oligonucleotides. Cationic lipids are utilized to effectively introduce polyanionic oligonucleotides and plansmid DNA into the intracellular space. For example, lipofectin, a liposome formulation of a cationic lipid has been used in various studies to transfect oligonucleotides into target cells. See e.g., J.K. Rose et al., 1991, Biotechniques, 10(4): 520-5; C.F. Beggett et al., 1992, Mol. Pharmacol. (41): 1023-1033; A. Colige et al., 1993, Biochemistry (32): 7-11; N.M. Dean et al., 1994, J. Biol. Chem. (269): 16416-16424; R.W. Wager et al., 1993, Science (260): 1510-1513. However, lipofectin is serum-sensitive; optimal transfection using lipofectin must be performed with serum-free conditions. See supra, J.G. Lewis et al. Lipofecin is also limited in the number of cell types it may effectively transfect. Other examples of liposomal transfection agents are cytofectin agents, which are cationic amphiphiles or cationic liposomes. They have been used to transfect DNA molecules into various cells. See e.g., J.H. Felgner et al., 1994, J. Biol. Chem. (269): 2550-2561; X. Gao and L. Huang, 1991, Biochem. Biophys. Res. Commun. (179): 280-285. GS2888 cytofectin, a cationic lipid formulated with the fusogenic lipid dioleoylphosphatidylethanolamine, was shown to significantly improve the transfection efficiency of antisense oligonucleotides as well as plasmid DNA. See supra, J.G. Lewis et al.

[0108] However, liposomal transfection agents such as lipofectin and cytofectin exert cytotoxic effects when used to transfect primary bone marrow-derived osteoclasts

cells. High concentrations of oligonucleotides, with or without lipid-based transfection agents, also result in cytotoxicity. Therefore, non-liposomal transfection agents are used to transfect oligonucleotide compounds into osteoclasts or osteoclast precursor cells according to various embodiments of the present disclosure.

[0109] FuGENE 6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN) is a non-liposomal formulation capable of transfecting eukaryotic cells with reasonable efficiency and low cytotoxicity. See, FuGENE 6 Transfection Reagent Instruction Manual, version 5, September 2000, Roche Diagnostics Corp. In one embodiment, oligonucleotide compounds such as antisense oligonucleotides for RANK are delivered into bone marrow derived osteoclast precursor cells in the presence of FuGENE 6. See infra Example 20. The antisense oligonucleotides were shown to inhibit the expression of RANK, i.e., decrease the RANK mRNA levels. See id. Inhibition on osteoclast differentiation was observed in bone marrow derived precursor cells upon transfection of the antisense oligonucleotides. See infra Example 21. More efficient inhibition of osteoclast differentiation is achieved when transfection is performed during early differentiation of cultured precursor cells, i.e., the early stage of the culturing. See infra Example 22. The early stage is after day two and before day four of the culturing. See id. See also, Example 9 infra. By day four, most of the cultured cells became matured osteoclasts. The methods of the present disclosure may be used, therefore, to deliver or transfect oligonucleotide compounds into osteoclast precursor cells in one embodiment and osteoclasts in another embodiment. In alternative embodiments, the methods may be used to transfect osteoclast-like cells in a cell line, such as RAW264.7

cells. RAW264.7 is a macrophage-like cell line that may be differentiated into an osteoclast phenotype in the presence of RANKL. See infra, Example 9.

[0110] Examples of other suitable non-liposomal transfection reagents include Effectene®, Calcium Phosphate, and DEAE-Dextran. See, Effectene® Transfection Reagent Handbook, 2002, Qiagen Inc. (www1.qiagen.com/literature/handbooks/PDF/Transfection/TF_Effectene/1020615HB_EF_0402WW.pdf).

[0111] Cellular delivery of oligonucleotide compounds into osteoclasts, osteoclast-precursor cells, and osteoclast-like cells, enables studies of osteoclast differentiation and other cellular activities in osteoclast cells. Introduction of these compounds into osteoclast cells allows modulation of osteoclast differentiation. Modulation according to various embodiments means regulation and control, i.e., inhibition or stimulation. For example, antisense RANK oligonucleotides are capable of inhibiting osteoclast differentiation upon transfection into osteoclast precursor cells. See infra, Example 21. Given the inhibitive effect of transfected antisense RANK oligonucleotides on differentiation of osteoclast cells, the methods of cellular delivery according to the present disclosure may be useful in devising therapeutics and diagnostics for bone abnormalities associated with osteoclastic activities. For example, various modulators or compounds that are capable of regulating osteoclast activities or bone metabolisms may be introduced according to the disclosed methods into osteoclasts or bone marrow precursor cells of a patient. These compounds or modulators may regulate the bone metabolisms in the patient and thereby treating the imbalances in osteoclastic and

osteoblastic activities. See supra, for more detailed discussions on diagnostics and therapeutics associated with antisense RANK oligonucleotides.

[0112] The present disclosure provides various compounds that may be used in conjunction with the cellular delivery methods. See supra for the detailed discussion in the subsection Compounds. For example, the compound may be 8 or 80 nucleobases in length. According to certain embodiments, the compound may be 12 to 50 nucleobases in length, and in alternative embodiments, the compound may be 15 to 30 nucleobases in length. The compound is an antisense oligonucleotide in one embodiment. In another embodiment, the compound is a DNA oligonucleotide. In yet another embodiment, the compound is a RNA oligonucleotide. The compound is a chimeric oligonucleotide in still another embodiment. In a further embodiment, at least a portion of the compound hybridizes with RNA to form an oligonucleotide-RNA duplex.

[0113] For antisense RANK oligonucleotide compounds, different levels of complementarity are provided in various embodiments. In one embodiment, the compound is at least 70% complementary to the region of the nucleic acid molecule encoding RANK. In another embodiment, the compound is at least 80% complementary to the region of the nucleic acid molecule encoding RANK. In yet another embodiment, the compound is at least 90% complementary to the region of the nucleic acid molecule encoding RANK. In still another embodiment, the compound is at least 95% complementary to the region of the nucleic acid molecule encoding RANK. In a further embodiment, the compound is at least 99% complementary to the region of the nucleic acid molecule encoding RANK.

[0114] The following examples further describe the various embodiments. They are illustrative of the disclosed embodiments but do not limit the same in any manner.

EXAMPLES

Example 1: Synthesis of Nucleoside Phosphoramidites

[0115] The following compounds, including amidites and their intermediates were prepared as described in US Patent 6,426,220 and published PCT WO 02/36743; 5'-O-Dimethoxytrityl-thymidine intermediate for 5-methyl dC amidite, 5'-O-Dimethoxytrityl-2'-deoxy-5-methylcytidine intermediate for 5-methyl-dC amidite, 5'-O-Dimethoxytrityl-2'-deoxy-N4-benzoyl-5-methylcytidine penultimate intermediate for 5-methyl dC amidite, [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-deoxy-N^4-benzoyl-5-methylcytidin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (5-methyl dC amidite), 2'-Fluorodeoxyadenosine, 2'-Fluorodeoxyguanosine, 2'-Fluorouridine, 2'-Fluorodeoxycytidine, 2'-O-(2-Methoxyethyl) modified amidites, 2'-O-(2-methoxyethyl)-5-methyluridine intermediate, 5'-O-DMT-2'-O-(2-methoxyethyl)-5-methyluridine penultimate intermediate, [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-methoxyethyl)-5-methyluridin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (MOE T amidite), 5'-O-Dimethoxytrityl-2'-O-(2-methoxyethyl)-5-methylcytidine intermediate, 5'-O-dimethoxytrityl-2'-*O*-(2-methoxyethyl)-N^4-benzoyl-5-methyl-cytidine penultimate intermediate, [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-methoxyethyl)-N^4-benzoyl-5-methylcytidin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (MOE 5-Me-C amidite), [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-methoxyethyl)-N^6-benzoyladenosin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (MOE A

amdite), [5'-*O*-(4,4'-Dimethoxytriphenylmethyl)-2'-*O*-(2-methoxyethyl)-N^4-isobutyrylguanosin-3'-*O*-yl]-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (MOE G amidite), 2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-(dimethylaminooxyethyl) nucleoside amidites, 2'-(Dimethylaminooxyethoxy) nucleoside amidites, 5'-O-tert-Butyldiphenylsilyl-O^2-2'-anhydro-5-methyluridine , 5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine, 2'-O-([2-phthalimidoxy)ethyl]-5'-*t*-butyldiphenylsilyl-5-methyluridine , 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine, 5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N dimethylaminooxyethyl]-5-methyluridine, 2'-O-(dimethylaminooxyethyl)-5-methyluridine, 5'-O-DMT-2'-O-(dimethylaminooxyethyl)-5-methyluridine, 5'-O-DMT-2'-O-(2-N,N-dimethylaminooxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite], 2'-(Aminooxyethoxy) nucleoside amidites, N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite], 2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites, 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine, 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl)]-5-methyl uridine and 5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)-ethyl)]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite.

Example 2: Oligonucleotide and oligonucleoside synthesis

[0116] The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase

synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

[0117] Oligonucleotides: Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 394) using standard phosphoramidite chemistry with oxidation by iodine.

[0118] Phosphorothioates (P=S) are synthesized similar to phosphodiester oligonucleotides with the following exceptions: thiation was effected by utilizing a 10% w/v solution of 3,H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the oxidation of the phosphite linkages. The thiation reaction step time was increased to 180 sec and preceded by the normal capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (12-16 hr), the oligonucleotides were recovered by precipitating with >3 volumes of ethanol from a 1 M NH_4OAc solution. Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

[0119] Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

[0120] 3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

[0121] Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

[0122] Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

[0123] 3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

[0124] Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

[0125] Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

[0126] Oligonucleosides: Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

[0127] Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

[0128] Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 3: RNA Synthesis

[0129] In general, RNA synthesis chemistry is based on the selective incorporation of various protecting groups at strategic intermediary reactions. Although one of ordinary skill in the art will understand the use of protecting groups in organic synthesis, a useful class of protecting groups includes silyl ethers. In particular bulky silyl ethers are used to protect the 5′-hydroxyl in combination with an acid-labile orthoester protecting group on the 2′-hydroxyl. This set of protecting groups is then used with standard solid-phase synthesis technology. It is important to lastly remove the acid labile orthoester protecting group after all other synthetic steps. Moreover, the early use of the silyl protecting groups during synthesis ensures facile removal when desired, without undesired deprotection of 2′ hydroxyl.

[0130] Following this procedure for the sequential protection of the 5′-hydroxyl in combination with protection of the 2′-hydroxyl by protecting groups that are differentially removed and are differentially chemically labile, RNA oligonucleotides were synthesized.

[0131] RNA oligonucleotides are synthesized in a stepwise fashion. Each nucleotide is added sequentially (3′- to 5′-direction) to a solid support-bound oligonucleotide. The first nucleoside at the 3′-end of the chain is covalently attached to a solid support. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are added,

coupling the second base onto the 5′-end of the first nucleoside. The support is washed and any unreacted 5′-hydroxyl groups are capped with acetic anhydride to yield 5′-acetyl moieties. The linkage is then oxidized to the more stable and ultimately desired P(V) linkage. At the end of the nucleotide addition cycle, the 5′-silyl group is cleaved with fluoride. The cycle is repeated for each subsequent nucleotide.

[0132] Following synthesis, the methyl protecting groups on the phosphates are cleaved in 30 minutes utilizing 1 M disodium-2-carbamoyl-2-cyanoethylene-1,1-dithiolate trihydrate (S_2Na_2) in DMF. The deprotection solution is washed from the solid support-bound oligonucleotide using water. The support is then treated with 40% methylamine in water for 10 minutes at 55 °C. This releases the RNA oligonucleotides into solution, deprotects the exocyclic amines, and modifies the 2′- groups. The oligonucleotides can be analyzed by anion exchange HPLC at this stage.

[0133] The 2′-orthoester groups are the last protecting groups to be removed. The ethylene glycol monoacetate orthoester protecting group developed by Dharmacon Research, Inc. (Lafayette, CO), is one example of a useful orthoester protecting group which, has the following important properties. It is stable to the conditions of nucleoside phosphoramidite synthesis and oligonucleotide synthesis. However, after oligonucleotide synthesis the oligonucleotide is treated with methylamine which not only cleaves the oligonucleotide from the solid support but also removes the acetyl groups from the orthoesters. The resulting 2-ethyl-hydroxyl substituents on the orthoester are less electron withdrawing than the acetylated precursor. As a result, the modified orthoester becomes more labile to acid-catalyzed hydrolysis. Specifically, the rate of cleavage is

approximately 10 times faster after the acetyl groups are removed. Therefore, this orthoester possesses sufficient stability in order to be compatible with oligonucleotide synthesis and yet, when subsequently modified, permits deprotection to be carried out under relatively mild aqueous conditions compatible with the final RNA oligonucleotide product.

[0134] Additionally, methods of RNA synthesis are well known in the art (Scaringe, S. A. Ph.D. Thesis, University of Colorado, 1996; Scaringe, S. A., et al., *J. Am. Chem. Soc.*, **1998**, *120*, 11820-11821; Matteucci, M. D. and Caruthers, M. H. *J. Am. Chem. Soc.*, **1981**, *103*, 3185-3191; Beaucage, S. L. and Caruthers, M. H. *Tetrahedron Lett.*, **1981**, *22*, 1859-1862; Dahl, B. J., et al., *Acta Chem. Scand,*. **1990**, *44*, 639-641; Reddy, M. P., et al., *Tetrahedrom Lett.*, **1994**, *25*, 4311-4314; Wincott, F. et al., *Nucleic Acids Res.*, **1995**, *23*, 2677-2684; Griffin, B. E., et al., *Tetrahedron,* **1967**, *23*, 2301-2313; Griffin, B. E., et al., *Tetrahedron,* **1967**, *23*, 2315-2331).

[0135] RNA antisense compounds (RNA oligonucleotides) of the present invention can be synthesized by the methods herein or purchased from Dharmacon Research, Inc (Lafayette, CO). Once synthesized, complementary RNA antisense compounds can then be annealed by methods known in the art to form double stranded (duplexed) antisense compounds. For example, duplexes can be formed by combining 30 µl of each of the complementary strands of RNA oligonucleotides (50 uM RNA oligonucleotide solution) and 15 µl of 5X annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH pH 7.4, 2 mM magnesium acetate) followed by heating for 1 minute at 90°C, then 1 hour at

37°C. The resulting duplexed antisense compounds can be used in kits, assays, screens, or other methods to investigate the role of a target nucleic acid.

Example 4: Synthesis of Chimeric Oligonucleotides

[0136] Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[0137] **[2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides**

[0138] Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 394, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by incorporating coupling steps with increased reaction times for the 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite. The fully protected oligonucleotide is cleaved from the support and deprotected in

concentrated ammonia (NH_4OH) for 12-16 hr at 55°C. The deprotected oligo is then recovered by an appropriate method (precipitation, column chromatography, volume reduced *in vacuo* and analyzed spetrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[0139] [2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[0140] [2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidation with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

[0141] Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

Example 5: Design and screening of duplexed antisense compounds targeting RANK

[0142] In accordance with the present invention, a series of nucleic acid duplexes comprising the antisense compounds of the present invention and their complements can be designed to target RANK. The nucleobase sequence of the antisense strand of the duplex comprises at least a portion of an oligonucleotide in Table 1. The ends of the strands may be modified by the addition of one or more natural or modified nucleobases to form an overhang. The sense strand of the dsRNA is then designed and synthesized as the complement of the antisense strand and may also contain modifications or additions to either terminus. For example, in one embodiment, both strands of the dsRNA duplex would be complementary over the central nucleobases, each having overhangs at one or both termini.

[0143] For example, a duplex comprising an antisense strand having the sequence CGAGAGGCGGACGGGACCG and having a two-nucleobase overhang of deoxythymidine(dT) would have the following structure:

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cgagaggcggacgggaccgTT    Antisense Strand
||||||||||||||||||
TTgctctccgcctgccctggc    Complement
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[0144] RNA strands of the duplex can be synthesized by methods disclosed herein or purchased from Dharmacon Research Inc., (Lafayette, CO). Once synthesized, the complementary strands are annealed. The single strands are aliquoted and diluted to a concentration of 50 uM. Once diluted, 30 uL of each strand is combined with 15uL of a 5X solution of annealing buffer. The final concentration of said buffer is 100 mM

potassium acetate, 30 mM HEPES-KOH pH 7.4, and 2mM magnesium acetate. The final volume is 75 uL. This solution is incubated for 1 minute at 90°C and then centrifuged for 15 seconds. The tube is allowed to sit for 1 hour at 37°C at which time the dsRNA duplexes are used in experimentation. The final concentration of the dsRNA duplex is 20 uM. This solution can be stored frozen (-20°C) and freeze-thawed up to 5 times.

[0145] Once prepared, the duplexed antisense compounds are evaluated for their ability to modulate RANK expression.

[0146] When cells reached 80% confluency, they are treated with duplexed antisense compounds of the invention. For cells grown in 96-well plates, wells are washed once with 200 µL OPTI-MEM-1 reduced-serum medium (Gibco BRL) and then treated with 130 µL of OPTI-MEM-1 containing 12 µg/mL LIPOFECTIN (Gibco BRL) and the desired duplex antisense compound at a final concentration of 200 nM. After 5 hours of treatment, the medium is replaced with fresh medium. Cells are harvested 16 hours after treatment, at which time RNA is isolated and target reduction measured by RT-PCR.

Example 6: Oligonucleotide Isolation

[0147] After cleavage from the controlled pore glass solid support and deblocking in concentrated ammonium hydroxide at 55°C for 12-16 hours, the oligonucleotides or oligonucleosides are recovered by precipitation out of 1 M NH_4OAc with >3 volumes of ethanol. Synthesized oligonucleotides were analyzed by electrospray mass spectroscopy (molecular weight determination) and by capillary gel electrophoresis and judged to be at least 70% full length material. The relative amounts of phosphorothioate and

phosphodiester linkages obtained in the synthesis was determined by the ratio of correct molecular weight relative to the –16 amu product (+/-32 +/-48). For some studies oligonucleotides were purified by HPLC, as described by Chiang *et al.*, *J. Biol. Chem.* **1991**, *266*, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 7: Oligonucleotide Synthesis - 96 Well Plate Format

[0148] Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a 96-well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-diiso-propyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per standard or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

[0149] Oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried *in vacuo*. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

Example 8: Oligonucleotide Analysis – 96-Well Plate Format

[0150] The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96-well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

Example 9: Cell culture and oligonucleotide treatment

[0151] The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily determined by methods routine in the art, for example Northern blot analysis, ribonuclease protection assays, or RT-PCR.

T-24 cells:

[0152] The mouse transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen Corporation, Carlsbad, CA), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Invitrogen Corporation, Carlsbad, CA). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #353872) at a density of 7000 cells/well for use in RT-PCR analysis.

[0153] For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

[0154] The mouse lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen Corporation, Carlsbad, CA), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Invitrogen Corporation, Carlsbad, CA). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells:

[0155] Mouse neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville, MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville, MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

[0156] Mouse embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville, MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville, MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

RAW264.7 cells:

[0157] The mouse Abelson murine leukemia virus-induced tumor macrophage cell line was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). RAW 264.7 cells were routinely cultured in alpha-MEM (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen Corporation, Carlsbad, CA), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Invitrogen Corporation, Carlsbad, CA). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 24-well plates

(Falcon-353047) at a density of ~20,000 cells/cm^2 for use in antisense oligonucleotide transfection.

Primary mouse bone marrow-derived osteoclasts:

[0158] Primary mouse osteoclasts were prepared from the bone marrow of ~4-month old, female BALB/C mice purchased from Charles River Laboratories. Primary mouse bone marrow suspensions were routinely cultured in alpha-MEM media (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (Cat #SH30071.03) (Hyclone, Logan, UT), 50ug/ml Gentamicin Sulfate Solution (Irvine Scientific, Santa Ana, CA), 50ng/ml murine monocyte-colony stimulating factor (M-CSF) (R&D Systems, Minneapolis, MN) and 100ng/ml soluble human receptor activator of NF-kB ligand (shRANKL) (Peprotech, Rocky Hill, NJ). Culture media containing all supplements, including RANKL and MCSF, is replaced every 3 days. Cells were seeded onto 24-well plates (Falcon-353047) at a density of ~75,000 cells/cm^2 for use in antisense oligonucleotide transfection.

Treatment with antisense compounds:

[0159] When cells reached 65-75% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 100 µL OPTI-MEM™-1 reduced-serum medium (Invitrogen Corporation, Carlsbad, CA) and then treated with 130 µL of OPTI-MEM™-1 containing 3.75 µg/mL LIPOFECTIN™ (Invitrogen Corporation, Carlsbad, CA) and the desired concentration of oligonucleotide.

Cells are treated and data are obtained in triplicate. After 4-7 hours of treatment at 37°C, the medium was replaced with fresh medium. Cells were harvested 16-24 hours after oligonucleotide treatment.

[0160] The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control oligonucleotide at a range of concentrations. For mouse cells the positive control oligonucleotide is selected from either ISIS 13920 (**TCC**GTCATCGCT**CCTCAGGG**, SEQ ID NO: 1) which is targeted to human H-ras, or ISIS 18078, (**GTGCG**CGCGAGCCCG**AAATC**, SEQ ID NO: 2) which is targeted to mouse Jun-N-terminal kinase-2 (JNK2). Both controls are 2'-O-methoxyethyl gapmers (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone. For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCA**TTCTGCCCCC**AAGGA**, SEQ ID NO: 3, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control oligonucleotide that results in 80% inhibition of c-H-ras (for ISIS 13920), JNK2 (for ISIS 18078) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest concentration of positive control oligonucleotide that results in 60% inhibition of c-H-ras, JNK2 or c-raf mRNA is then utilized as the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is deemed as unsuitable for oligonucleotide

transfection experiments. The concentrations of antisense oligonucleotides used herein are from 50 nM to 300 nM.

Example 10: Analysis of oligonucleotide inhibition of RANK expression

[0161] Antisense modulation of RANK expression can be assayed in a variety of ways known in the art. For example, RANK mRNA levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). Real-time quantitative PCR is presently preferred. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. The preferred method of RNA analysis of the present invention is the use of total cellular RNA as described in other examples herein. Methods of RNA isolation are well known in the art. Northern blot analysis is also routine in the art. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISM™ 7600, 7700, or 7900 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

[0162] Protein levels of RANK can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), enzyme-linked immunosorbent assay (ELISA) or fluorescence-activated cell sorting (FACS). Antibodies directed to RANK can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional monoclonal or polyclonal antibody generation methods well known in the art.

Example 11: Design of phenotypic assays and *in vivo* studies for the use of RANK inhibitors

Phenotypic assays

[0163] Once RANK inhibitors have been identified by the methods disclosed herein, the compounds are further investigated in one or more phenotypic assays, each having measurable endpoints predictive of efficacy in the treatment of a particular disease state or condition.

[0164] Phenotypic assays, kits and reagents for their use are well known to those skilled in the art and are herein used to investigate the role and/or association of RANK in health and disease. Representative phenotypic assays, which can be purchased from any one of several commercial vendors, include those for determining cell viability, cytotoxicity, proliferation or cell survival (Molecular Probes, Eugene, OR; PerkinElmer, Boston, MA), protein-based assays including enzymatic assays (Panvera, LLC, Madison, WI; BD Biosciences, FRANKin Lakes, NJ; Oncogene Research Products, San Diego, CA), cell regulation, signal transduction, inflammation, oxidative processes and apoptosis (Assay Designs Inc., Ann Arbor, MI), triglyceride accumulation (Sigma-Aldrich, St. Louis, MO), angiogenesis assays, tube formation assays, cytokine and hormone assays and metabolic assays (Chemicon International Inc., Temecula, CA; Amersham Biosciences, Piscataway, NJ).

[0165] In one non-limiting example, cells determined to be appropriate for a particular phenotypic assay (i.e., MCF-7 cells selected for breast cancer studies;

adipocytes for obesity studies) are treated with RANK inhibitors identified from the *in vitro* studies as well as control compounds at optimal concentrations which are determined by the methods described above. At the end of the treatment period, treated and untreated cells are analyzed by one or more methods specific for the assay to determine phenotypic outcomes and endpoints.

[0166] Phenotypic endpoints include changes in cell morphology over time or treatment dose as well as changes in levels of cellular components such as proteins, lipids, nucleic acids, hormones, saccharides or metals. Measurements of cellular status which include pH, stage of the cell cycle, intake or excretion of biological indicators by the cell, are also endpoints of interest.

[0167] Analysis of the geneotype of the cell (measurement of the expression of one or more of the genes of the cell) after treatment is also used as an indicator of the efficacy or potency of the RANK inhibitors. Hallmark genes, or those genes suspected to be associated with a specific disease state, condition, or phenotype, are measured in both treated and untreated cells.

In vivo studies

[0168] The individual subjects of the *in vivo* studies described herein are warm-blooded vertebrate animals, which includes mouses.

[0169] The clinical trial is subjected to rigorous controls to ensure that individuals are not unnecessarily put at risk and that they are fully informed about their role in the study.

[0170] To account for the psychological effects of receiving treatments, volunteers are randomly given placebo or RANK inhibitor. Furthermore, to prevent the doctors from being biased in treatments, they are not informed as to whether the medication they are administering is a RANK inhibitor or a placebo. Using this randomization approach, each volunteer has the same chance of being given either the new treatment or the placebo.

[0171] Volunteers receive either the RANK inhibitor or placebo for eight week period with biological parameters associated with the indicated disease state or condition being measured at the beginning (baseline measurements before any treatment), end (after the final treatment), and at regular intervals during the study period. Such measurements include the levels of nucleic acid molecules encoding RANK or RANK protein levels in body fluids, tissues or organs compared to pre-treatment levels. Other measurements include, but are not limited to, indices of the disease state or condition being treated, body weight, blood pressure, serum titers of pharmacologic indicators of disease or toxicity as well as ADME (absorption, distribution, metabolism and excretion) measurements.

[0172] Information recorded for each patient includes age (years), gender, height (cm), family history of disease state or condition (yes/no), motivation rating (some/moderate/great) and number and type of previous treatment regimens for the indicated disease or condition.

[0173] Volunteers taking part in this study are healthy adults (age 18 to 65 years) and roughly an equal number of males and females participate in the study. Volunteers

with certain characteristics are equally distributed for placebo and RANK inhibitor treatment. In general, the volunteers treated with placebo have little or no response to treatment, whereas the volunteers treated with the RANK inhibitor show positive trends in their disease state or condition index at the conclusion of the study.

Example 12: RNA Isolation

Poly(A)+ mRNA isolation

[0174] Poly(A)+ mRNA was isolated according to Miura *et al.*, (*Clin. Chem.*, **1996**, *42*, 1758-1764). Other methods for poly(A)+ mRNA isolation are routine in the art. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 μL cold PBS. 60 μL lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 μL of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 μL of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 μL of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C, was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

[0175] Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

Total RNA Isolation

[0176] Total RNA was isolated using an RNEASY 96™ kit and buffers purchased from Qiagen Inc. (Valencia, CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 µL cold PBS. 150 µL Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 150 µL of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 1 minute. 500 µL of Buffer RW1 was added to each well of the RNEASY 96™ plate and incubated for 15 minutes and the vacuum was again applied for 1 minute. An additional 500 µL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum was applied for 2 minutes. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of 90 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 3 minutes. The plate was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then

eluted by pipetting 140 µL of RNAse free water into each well, incubating 1 minute, and then applying the vacuum for 3 minutes.

[0177] The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc., Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

Example 13: Real-time Quantitative PCR Analysis of RANK mRNA Levels

[0178] Quantitation of RANK mRNA levels was accomplished by real-time quantitative PCR using the ABI PRISM™ 7600, 7700, or 7900 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., FAM or JOE, obtained from either PE-Applied Biosystems, Foster City, CA, Operon Technologies Inc., Alameda, CA or Integrated DNA Technologies Inc., Coralville, IA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either PE-Applied Biosystems, Foster City, CA, Operon Technologies Inc., Alameda, CA or Integrated DNA Technologies Inc.,

Coralville, IA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

[0179] Prior to quantitative PCR analysis, primer-probe sets specific to the target gene being measured are evaluated for their ability to be "multiplexed" with a GAPDH amplification reaction. In multiplexing, both the target gene and the internal standard gene GAPDH are amplified concurrently in a single sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each dilution is amplified in the presence of primer-probe sets specific for GAPDH only, target gene only ("single-plexing"), or both (multiplexing). Following PCR amplification, standard curves of GAPDH and target mRNA signal as a function of dilution are generated from both the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their

corresponding values generated from the single-plexed samples, the primer-probe set specific for that target is deemed multiplexable. Other methods of PCR are also known in the art.

[0180] PCR reagents were obtained from Invitrogen Corporation, (Carlsbad, CA). RT-PCR reactions were carried out by adding 20 μL PCR cocktail (2.5x PCR buffer minus $MgCl_2$, 6.6 mM $MgCl_2$, 375 μM each of dATP, dCTP, dCTP and dGTP, 375 nM each of forward primer and reverse primer, 125 nM of probe, 4 Units RNAse inhibitor, 1.25 Units PLATINUM® Taq, 5 Units MuLV reverse transcriptase, and 2.5x ROX dye) to 96-well plates containing 30 μL total RNA solution (20-200 ng). The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the PLATINUM® Taq, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

[0181] Gene target quantities obtained by real time RT-PCR are normalized using either the expression level of GAPDH, a gene whose expression is constant, or by quantifying total RNA using RiboGreen™ (Molecular Probes, Inc. Eugene, OR). GAPDH expression is quantified by real time RT-PCR, by being run simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RiboGreen™ RNA quantification reagent (Molecular Probes, Inc. Eugene, OR). Methods of RNA quantification by RiboGreen™ are taught in Jones, L.J., et al, (Analytical Biochemistry, 1998, 265, 368-374).

[0182] In this assay, 170 μL of RiboGreen™ working reagent (RiboGreen™ reagent diluted 1:350 in 10mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 30 μL purified, cellular RNA. The plate is read in a CytoFluor 4000 (PE Applied Biosystems) with excitation at 485nm and emission at 530nm.

[0183] Probes and primers to mouse RANK were designed to hybridize to a mouse RANK sequence, using published sequence information (GenBank accession number NM_009399.1, incorporated herein as SEQ ID NO:4). For mouse RANK the PCR primers were:

forward primer: GGTCTGCAGCTCTTCCATGAC (SEQ ID NO: 5)
reverse primer: TGAGACTGGGCAGGTAAGCC (SEQ ID NO: 6) and the PCR probe was: FAM-TGAGGAGACCACCCAAGGAGGCC-TAMRA
(SEQ ID NO: 7) where FAM is the fluorescent dye and TAMRA is the quencher dye. For mouse GAPDH the PCR primers were:
forward primer: GGCAAATTCAACGGCACAGT (SEQ ID NO:8)
reverse primer: GAAGATGGTGATGGGATTTC (SEQ ID NO:9) and the PCR probe was: 5' JOE-AAGGCCGAGAATGGGAAGCTTGTCATC- TAMRA 3' (SEQ ID NO: 10) where JOE is the fluorescent reporter dye and TAMRA is the quencher dye.

Example 14: Northern blot analysis of RANK mRNA levels

[0184] Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was prepared following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc. Solon,

OH). RNA was transferred from the gel to HYBOND™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, La Jolla, CA) and then probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions.

[0185] To detect mouse RANK, a mouse RANK specific probe was prepared by PCR using the forward primer GGTCTGCAGCTCTTCCATGAC (SEQ ID NO: 5) and the reverse primer TGAGACTGGGCAGGTAAGCC (SEQ ID NO: 6). To normalize for variations in loading and transfer efficiency membranes were stripped and probed for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto, CA).

[0186] Hybridized membranes were visualized and quantitated using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3 (Molecular Dynamics, Sunnyvale, CA). Data was normalized to GAPDH levels in untreated controls.

Example 15: Antisense inhibition of mouse RANK expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

[0187] In accordance with the present invention, a series of antisense compounds was designed to target different regions of the mouse RANK RNA, using published sequences (GenBank accession number NM_009399.1, incorporated herein as SEQ ID

NO: 4). The compounds are shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the compound binds. All compounds in Table 1 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds can be analyzed for their effect on mouse RANK mRNA levels by quantitative real-time PCR as described in other examples herein.

Table 1

Inhibition of mouse RANK mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	% INHIB	SEQ ID NO
181048	start codon	4	8	CATGGCGCGGTGCGGGCGGG	55	11
181049	start codon	4	11	GGCCATGGCGCGGTGCGGGC	65	12
181050	start codon	4	12	GGGCCATGGCGCGGTGCGGG	64	13
181051	coding	4	57	GCGCCAGCAGCGGCGCGGGC	66	14
181052	coding	4	161	TTCGCATCTGCTGCAACACC	79	15
181053	coding	4	253	TCATTCCAGGTGTCCAAGTA	70	16
181054	coding	4	268	AAGCATTTATCTTCTTCATT	53	17
181055	coding	4	277	TTATGCAGCAAGCATTTATC	46	18
181056	coding	4	352	GTGCAAGCACAGCGACGCGG	76	19
181057	coding	4	358	CCAGCCGTGCAAGCACAGCG	67	20
181058	coding	4	403	TCCGTGTTCCTGCGGCAGCA	47	21
181059	coding	4	484	AAGAAGCCCAGGAGGCAGGG	60	22
181060	coding	4	582	CATCTGATTCCGTTGTCCCC	63	23
181061	coding	4	617	TGGTCTCCTCAGTGTCATGG	73	24
181062	coding	4	642	GCAGGTAAGCCTGGGCCTCC	71	25
181063	coding	4	711	AGTAAACGCCGAAGATGATG	60	26

181064	coding	4	717	TCCTGTAGTAAACGCCGAAG	54	27
181065	coding	4	733	AGCGCTTTCCCTCCCTTCCT	58	28
181066	coding	4	788	ATTTCCACTTAGACTACTGC	45	29
181067	coding	4	876	GAGTCATTAGTAAGATACCT	54	30
181068	coding	4	879	CCCGAGTCATTAGTAAGATA	61	31
181069	coding	4	936	CTGCCGCACACACAGGCCCA	59	32
181070	coding	4	949	GCCCAGGGCCCACCTGCCGC	66	33
181071	coding	4	1017	TCCTCGAGAGGTCTCCTTGC	75	34
181072	coding	4	1061	AGGCTGCGAGGGCCGGTCCG	62	35
181073	coding	4	1144	TCGTTCTCCCCCACTTCCAG	50	36
181074	coding	4	1196	GCCCTCAGAATCCACCGTGC	75	37
181075	coding	4	1263	TTGTCAGGTGCTTTTCAGGG	62	38
181076	coding	4	1279	TCACCTTCTATTTCTTTTGT	66	39
181077	coding	4	1353	CCTCCCCAGGAGTGTTCCCA	62	40
181078	coding	4	1443	TGCTGGCTGCTGCTTCACTG	43	41
181079	coding	4	1464	GCCGTACTCCCGCCTCTGCC	58	42
181080	coding	4	1510	GAGCTCCCGGACCCTGAGGC	75	43
181081	coding	4	1561	GAGTTACTGTTTCCAGTCAC	59	44
181082	coding	4	1567	AACGTGGAGTTACTGTTTCC	60	45
181083	coding	4	1597	TTGAAGTTCATCACCTGCCC	54	46
181084	coding	4	1642	CCCTCCTGCGAGGTCTGGCT	59	47
181085	coding	4	1678	CCCACGGGCTCCGACTCGGG	52	48
181086	coding	4	1692	CCTGCACAGGGCGGCCCACG	74	49
181087	coding	4	1735	GGCGCGGTGCCCGCAAAGGA	46	50
181088	coding	4	1763	CCCGGTGGCACAGACGTCGG	42	51
181089	coding	4	1822	TGCACCGGCCGCGATGTCCC	49	52
181090	coding	4	1847	TGAAGTCTGCGCCCCACCCT	40	53
181091	3'UTR	4	1911	GCACCCAGGGCAGACAGAGA	17	54
181092	3'UTR	4	1931	TGGAAAGGCACTGGTGCCCT	54	55
181093	3'UTR	4	1986	TGCCAGCAGCCTGCACCAGT	38	56
181094	3'UTR	4	2003	GGTGGGCTCCATCACCATGC	57	57
181095	3'UTR	4	2066	AGGCCAAACTGAATGATGCC	63	58

[0188] As shown in Table 1, SEQ ID Nos 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 55, 56, 57, and 58 demonstrated at least 35% inhibition of mouse RANK expression in this experiment and are therefore preferred. More preferred are SEQ ID Nos 19, 34 and 43. The target regions to which these preferred sequences are complementary are herein referred to as "preferred target segments" and are therefore preferred for targeting by compounds of the present invention. These preferred target

segments are shown in Table 2. These sequences are shown to contain thymine (T) but one of skill in the art will appreciate that thymine (T) is generally replaced by uracil (U) in RNA sequences. The sequences represent the reverse complement of the preferred antisense compounds shown in Table 1. "Target site" indicates the first (5'-most) nucleotide number on the particular target nucleic acid to which the oligonucleotide binds. Also shown in Table 2 is the species in which each of the preferred target segments was found.

Table 2

Sequence and position of preferred target segments identified in RANK.

Site ID	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	REV COMP OF SEQ ID	ACTIVE IN	SEQ ID NO
96217	4	8	CCCGCCCGCACCGCGCCATG	11	*M. musculus*	59
96218	4	11	GCCCGCACCGCGCCATGGCC	12	*M. musculus*	60
96219	4	12	CCCGCACCGCGCCATGGCCC	13	*M. musculus*	61
96220	4	57	GCCCGCGCCGCTGCTGGCGC	14	*M. musculus*	62
96221	4	161	GGTGTTGCAGCAGATGCGAA	15	*M. musculus*	63
96222	4	253	TACTTGGACACCTGGAATGA	16	*M. musculus*	64
96223	4	268	AATGAAGAAGATAAATGCTT	17	*M. musculus*	65
96224	4	277	GATAAATGCTTGCTGCATAA	18	*M. musculus*	66
96225	4	352	CCGCGTCGCTGTGCTTGCAC	19	*M. musculus*	67
96226	4	358	CGCTGTGCTTGCACGGCTGG	20	*M. musculus*	68
96227	4	403	TGCTGCCGCAGGAACACGGA	21	*M. musculus*	69
96228	4	484	CCCTGCCTCCTGGGCTTCTT	22	*M. musculus*	70
96229	4	582	GGGGACAACGGAATCAGATG	23	*M. musculus*	71
96230	4	617	CCATGACACTGAGGAGACCA	24	*M. musculus*	72
96231	4	642	GGAGGCCCAGGCTTACCTGC	25	*M. musculus*	73
96232	4	711	CATCATCTTCGGCGTTTACT	26	*M. musculus*	74
96233	4	717	CTTCGGCGTTTACTACAGGA	27	*M. musculus*	75
96234	4	733	AGGAAGGGAGGGAAAGCGCT	28	*M. musculus*	76
96235	4	788	GCAGTAGTCTAAGTGGAAAT	29	*M. musculus*	77
96236	4	876	AGGTATCTTACTAATGACTC	30	*M. musculus*	78
96237	4	879	TATCTTACTAATGACTCGGG	31	*M. musculus*	79
96238	4	936	TGGGCCTGTGTGTGCGGCAG	32	*M. musculus*	80

96239	4	949	GCGGCAGGTGGGCCCTGGGC	33	*M. musculus*	81
96240	4	1017	GCAAGGAGACCTCTCGAGGA	34	*M. musculus*	82
96241	4	1061	CGGACCGGCCCTCGCAGCCT	35	*M. musculus*	83
96242	4	1144	CTGGAAGTGGGGGAGAACGA	36	*M. musculus*	84
96243	4	1196	GCACGGTGGATTCTGAGGGC	37	*M. musculus*	85
96244	4	1263	CCCTGAAAAGCACCTGACAA	38	*M. musculus*	86
96245	4	1279	ACAAAAGAAATAGAAGGTGA	39	*M. musculus*	87
96246	4	1353	TGGGAACACTCCTGGGGAGG	40	*M. musculus*	88
96247	4	1443	CAGTGAAGCAGCAGCCAGCA	41	*M. musculus*	89
96248	4	1464	GGCAGAGGCGGGAGTACGGC	42	*M. musculus*	90
96249	4	1510	GCCTCAGGGTCCGGGAGCTC	43	*M. musculus*	91
96250	4	1561	GTGACTGGAAACAGTAACTC	44	*M. musculus*	92
96251	4	1567	GGAAACAGTAACTCCACGTT	45	*M. musculus*	93
96252	4	1597	GGGCAGGTGATGAACTTCAA	46	*M. musculus*	94
96253	4	1642	AGCCAGACCTCGCAGGAGGG	47	*M. musculus*	95
96254	4	1678	CCCGAGTCGGAGCCCGTGGG	48	*M. musculus*	96
96255	4	1692	CGTGGGCCGCCCTGTGCAGG	49	*M. musculus*	97
96256	4	1735	TCCTTTGCGGGCACCGCGCC	50	*M. musculus*	98
96257	4	1763	CCGACGTCTGTGCCACCGGG	51	*M. musculus*	99
96258	4	1822	GGGACATCGCGGCCGGTGCA	52	*M. musculus*	100
96259	4	1847	AGGGTGGGGCGCAGACTTCA	53	*M. musculus*	101
96261	4	1931	AGGGCACCAGTGCCTTTCCA	55	*M. musculus*	102
96262	4	1986	ACTGGTGCAGGCTGCTGGCA	56	*M. musculus*	103
96263	4	2003	GCATGGTGATGGAGCCCACC	57	*M. musculus*	104
96264	4	2066	GGCATCATTCAGTTTGGCCT	58	*M. musculus*	105

[0189] As these "preferred target segments" have been found by experimentation to be open to, and accessible for, hybridization with the antisense compounds of the present invention, one of skill in the art will recognize or be able to ascertain, using no more than routine experimentation, further embodiments of the invention that encompass other compounds that specifically hybridize to these preferred target segments and consequently inhibit the expression of RANK.

[0190] According to the present invention, antisense compounds include antisense oligomeric compounds, antisense oligonucleotides, ribozymes, external guide sequence (EGS) oligonucleotides, alternate splicers, primers, probes, and other short oligomeric compounds which hybridize to at least a portion of the target nucleic acid.

Example 16: Western blot analysis of RANK protein levels

[0191] Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to RANK is used, with a radiolabeled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

Example 17: Antisense inhibition of mouse RANK expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap: dose response study

[0192] In accordance with the present invention, a subset of the antisense oligonuclotides in Example 15 was further investigated in dose response studies.

[0193] ISIS 29848 (NNNNNNNNNNNNNNNNNNNN, where N = A, T, C or G; SEQ ID NO: 106) was used as a control oligonucleotide. ISIS 29848 is a chimeric oligonucleotide ("gapmer") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-

MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.

[0194] Treatment doses were 100, 200, and 400 nM. ISIS 181056 (SEQ ID NO: 19), 181071 (SEQ ID NO: 34), 181080 (SEQ ID NO: 43), and 181086 (SEQ ID NO: 49) and the control oligonucleotide ISIS 29848 were analyzed for their effect on mouse RANK mRNA levels in RAW 264.7 cells by quantitative real-time PCR as described in other examples herein. Data, shown in Table 3, are averages from three experiments and demonstrate that ISIS 181056, ISIS 181071, and ISIS 181080 were able to reduce RANK mRNA expression in a dose-dependent manner.

Table 3

Antisense inhibition of mouse RANK expression by chimeric phosphorothioate oligonucleotides have 2''MOE wings and a deoxy gap: dose response

	Dose (nM)		
	100	200	400
ISIS #	% Inhibition		
181056	42	49	56
181071	32	44	68
181080	43	54	58
181086	42	47	35
29848	0	0	0

Example 18: Specificity of RANK antisense oligonucleotides: loss of potency with increasing number of mismatched bases

[0195] In accordance with the present invention, the specificity of ISIS 181071 (SEQ ID NO: 34) as an inhibitor of RANK expression was evaluated. ISIS 181071 (SEQ

ID NO: 34) was compared with oligonucleotides targeted to the same nucleotide region, but which contained 2, 4, 6, or 8 mismatches.

[0196] The sequences of the mismatch oligonucleotides are, respectively, TCCTCGAGTGATCTCCTTGC (ISIS 208562, SEQ ID NO: 107), TCCTCGACTGATTTCCTTGC (ISIS 208563, SEQ ID NO: 108), TCCTCAACTGATTTGCTTGC (ISIS 208564, SEQ ID NO: 109), and TCATCAACTGATTTGCTTGT (ISIS 208565, SEQ ID NO: 110). ISIS 208562, ISIS 208563, ISIS 208564 and ISIS 208565 are chimeric oligonucleotides ("gapmer") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines.

[0197] The compounds were analyzed for their effect on mouse RANK mRNA levels in RAW264.7 cells by quantitative real-time PCR as described in other examples herein. Data, shown in Table 4, are averages from three experiments and show that the inhibition of RANK by the oligonucleotide of the present invention is specific to the antisense oligonucleotide sequence.

Table 4

Specificity of ISIS 181071: loss of inhibition with increasing number of mismatatched bases

ISIS No.	# Mismatch	% Inhib
Untreated	n.a.	0
181071	0	91
208562	2	68
208563	4	21
208564	6	24
208565	8	15

Example 19: Time course of RANK mRNA inhibition in a mouse macrophage cell line

[0198] In accordance with the present invention, unstimulated cells from a mouse macrophage cell line, designated RAW264.7 cells, were treated with ISIS 181071 (SEQ ID NO: 34) or 181080 (SEQ ID NO: 43) for varying amounts of time to assess the inhibition of RANK mRNA expression. The cells were treated with either ISIS 181071 or 181080 or with the control oligonucleotide ISIS 29848 (SEQ ID NO: 106) for 0, 2, 4, 8, 12, 24, 48, 72, 120, or 144 hours. The compounds were analyzed for their effect on mouse RANK mRNA levels in RAW264.7 cells by quantitative real-time PCR as described in other examples herein. The results are expressed as percent of mRNA inhibition relative to untreated control cells. Data, shown in Table 5, are averages from three experiments and illustrate that antisense inhibition of mouse RANK by the oligonucleotides of the present invention persists for at least 72 hours in cultured cells.

Table 5

Inhibition of mouse RANK in unstimulated RAW 264.7 cells: time course study

	% Inhibition of RANK mRNA		
Time (hours)	ISIS 181071	ISIS 181080	ISIS 29848
Untreated	0	0	0
2	91	0	0
4	99	44	2
8	99	49	6
12	98	55	0
24	98	48	0
48	87	57	0
72	96	79	9
120	0	0	0
144	0	0	0

Example 20: FuGENE 6-mediated introduction of RANK antisense oligonucleotides into mouse bone-marrow derived osteoclasts: dose response study

[0199] Transfection of nucleic acids into primary osteoclasts has, to date, not been reported in the art. In accordance with the present invention, a method of introducing antisense oligonucleotides into primary mouse osteoclasts was evaluated. Primary osteoclasts are isolated from mouse bone marrow. Approximately 4-month old, female BALB/C mice, purchased from Charles River Laboratories (Wilmington, MA), are sacrificed and the long bones are removed. A needle is inserted into the bone marrow compartment, and a syringe is used to flow saline through the bone marrow. The material flushed from the bone marrow is collected and filtered such that a single cell suspension of bone marrow-derived osteoclasts is obtained. These cells are immediately placed into 24-well culture dishes (Falcon #353047, BD Biosciences, Bedford, MA) at a density of 150,000 cells per well. Each well contains 400 μl of alpha-MEM (Invitrogen

Corporation, Carlsbad, CA). Cell culture supplements are added as described in other examples herein and include 50 ng/mL RANK-ligand (RANKL) and 50 ng/mL macrophage colony stimulating factor (MCSF).

[0200] Differentiation of cultured primary osteoclasts requires two successive treatments of RANKL and MCSF, with proliferation preferentially occuring after the first application and with differentiation occuring only after the second application of these agents. The first application occurs when the freshly isolated bone marrow-derived cells are placed into the supplemented culture media, and the second application occurs 3 days later upon removal and subsequent replacement of the culture media. The mechanisms by which osteoclast precursors differentiate into mature, bone degrading osteoclasts can be investigated by introducing into the undifferentiated osteoclasts the antisense oligonucleotides of the present invention and subsequently inducing differentiation by the addition of RANKL and MCSF.

[0201] FuGENE 6 Transfection Reagent (Roche Diagnostics Corp., Indianapolis, IN), a non-liposomal formulation for the transfection of eukaryotic cells, was used to deliver antisense oligonucleotides into mouse primary bone marrow-derived osteoclasts. The FuGENE 6 transfection reagent and oligonucleotides are added to polystyrene tubes containing serum-free alpha-MEM. The volume of FuGENE 6 is kept constant at 4 µl, while the concentration of oligonucleotide varies according to the design of the experiment. The volume of the serum-free media is adjusted such that the total volume of the serum-free media, FuGENE 6 and oligonucleotide mixture is 100 µl. This mixture is then added to the primary osteoclast precursor cells in the 24-well plate. The

transfection reagent was added without replacement of the existing culture media with a fresh media. The cells that are non-adherent thus remain in the media and may be transfected as well. The effect of transfected RANK oligo in inhititing osteoclast differentiation of these cells may be enhanced because the precursor cells may be transfected before being induced to differentiate into osteoclasts by RANKL and MCSF present in a fresh media (i.e. a second application of RANKL and MCSF).

[0202] A subset of the antisense oligonuclotides in Example 15 was further investigated in dose-response studies. The oligonucleotides ISIS 181071 (SEQ ID NO: 34) and the 8 base-pair mismatch ISIS 208565 (SEQ ID NO: 110) were analyzed for their effect on mouse RANK mRNA levels in primary mouse bone-marrow derived osteoclasts. Bone marrow cells were treated with 50 ng/mL each of RANKL and MCSF to induce osteoclast differentiation (i.e. a second application of RANKL and MCSF). After 48 hours, cells were transfected with 10, 50, 150 or 250 nM doses of oligonucleotide in the presence of FuGENE 6 (4 µl per well) and cultured for an additional 48 hours. The levels of mouse RANK mRNA were measured by quantitative real-time PCR as described in other examples herein. Data, shown in Table 6, are expressed as percent inhibition relative to untreated control cells and are the average from four experiments. The data illustrate that FuGENE 6 is capable of introducing the antisense oligonucleotides of the present invention into primary osteoclasts, and that the resultant inhibition of mouse RANK mRNA expression occurs in a dose dependent manner.

Table 6

FuGENE-mediated introduction of RANK antisense oligonucleotides into mouse bone-marrow derived osteoclasts: dose response study

	% RANK mRNA inhibition	
FuGENE6	14	
Untreated	0	
Oligonucleotide Dose (nM)	ISIS No.	
	181071	208565
10	32	5
50	46	9
100	77	12
250	93	34

Example 21: Antisense inhibition of osteoclast differentiation from bone marrow precursor cells: dose response study

[0203] In accordance with the present invention, a subset of the antisense oligonucleotides in Example 15 was further investigated to evaluate the relationship between antisense oligonucleotide dose and inhibition of osteoclast differentiation. The maturation of primary bone marrow-derived osteoclast precursors into osteoclasts can be measured by the appearance of tartrate-resistant acid phosphatase (TRAP)-positive and multinucleated cells, two phenotypes which are indicative of osteoclast differentiation.

[0204] The oligonucleotides ISIS 181071 (SEQ ID NO: 34) and the 8 base-pair mismatch ISIS 208565 (SEQ ID NO: 110) were analyzed for their effect on inhibiting the formation of TRAP-positive, multinucleated cells from primary bone-marrow cells. Bone marrow cells were treated with 50 ng/mL each of RANKL and MCSF to induce

osteoclast differentiation (i.e. a second application of RANKL and MCSF). After 48 hours, the cells were transfected with 10, 50, 150 or 250 nM doses of oligonucleotide in the presence of FuGENE 6 (Roche Diagnostics Corp., Indianapolis, IN) for 24 hours. After an additional 24 hours, cultures were evaluated for osteoclast differentiation. The data are the average of four experiments and are summarized in Table 7. The results illustrate that the oligonucleotide of the present invention is capable of inhibiting osteoclast differentiation in a dose-dependent manner.

Table 7

Inhibition of mouse osteoclast differentiation by RANK antisense oligonucleotide: dose response study

	Number of TRAP+, multinucleated cells/well	
Untreated	549	
FuGENE 6 only	425	
Oligonucleotide dose, nM	ISIS 181071	ISIS 208565
10	599	672
50	453	606
100	266	626
250	116	442

Example 22: More efficient inhibition of osteoclast formation with early antisense transfection

[0205] In accordance with the present invention, the efficiency of antisense oligonucleotide inhibition of osteoclast development as a function of cultured cell age was evaluated. Mouse primary bone marrow cells were divided into two populations. Both populations were treated with RANKL and MCSF to induce differentiation (i.e. a

second application of RANKL and MCSF). One population was transfected two days following induction of differentiation, and the second population was transfected four days following induction of differentiation. Cells were treated with saline, FuGENE 6 (Roche Diagnostics Corp., Indianapolis, IN) alone or with oligonucleotides, ISIS 181071 (SEQ ID NO: 34) or control oligonucleotide ISIS 208565 (SEQ ID NO: 110), in the presence of FuGENE 6. Cells differentiated for two days were evaluated two and three days after oligonucleotide or control treatment. Cells differentiated for four days were evaluated one and three days after oligonucleotide or control treatment. At the end of the transfection period, cultures were scored for the presence of multinucleated, TRAP-positive cells. The data are presented in Table 8 as the number of TRAP-positive cells, or differentiated osteoclasts, per dose of oligonucleotide. The data show that introduction of an antisense oligonucleotide of the present invention is more efficient as an inhibitor of osteoclast differentiation when introduced on the second day, rather than the fourth day, of osteoclast culture.

Table 8

Inhibition of osteoclast differentiation by early FuGENE-mediated transfection of antisense oligonucleotide

	TRAP+, multinucleated cells per field			
	Transfection on day 2 of differentiation		Transfection on day 4 of differentiation	
	Days following transfection			
	2	3	1	3
Untreated	15	47	52	54
FuGENE 6	9	39	59	58
181071	0	6	42	39
208565	9	25	54	61

Example 23: Caspase 3 activity in primary mouse bone marrow-derived osteoclasts

[0206] Caspase 3 activation is an early marker of apoptosis. RANK affords protection from apoptosis, so the effects of RANK antisense oligonucleotide treatment on osteoclast apoptosis were evaluated by examining caspase 3 activity in fully differentiated osteoclasts.

[0207] In accordance with the present invention, primary mouse bone marrow-derived cells were treated with 50 ng/mL each of RANKL and MCSF to induce osteoclast differentiation (i.e. a second application of RANKL and MCSF). After 48 hours, these bone marrow-derived osteoclasts were treated with 300nM ISIS 181071 (SEQ ID NO: 34) or ISIS 208565 (SEQ ID NO: 110), in the presence of FuGENE 6, for either 2 or 4 hours and caspase 3 activity was measured. Caspase 3 activity is expressed as the percentage of activity relative to the untreated control. The data are summarized in Table 9 and show that treatment with the oligonucleotide of the present invention for 4 hours substantially increases caspase 3 activity, indicating that the oligonucleotide is able to interfere with RANK activity.

Table 9

Caspase 3 activity in primary mouse bone marrow-derived osteoclasts transfected with mouse RANK antisense oligonucleotides

	Isis No.	
	181071	208565
Time	caspase 3 activity (% of untreated control)	
2hr	96	74
4hr	332	145

[0208] This and other examples herein illustrate that FuGENE 6 is an effective agent for the delivery of antisense oligonucleotides into osteoclasts, a method that is to data not reported in the art. These data also demonstrate that FuGENE 6-mediated transfection of antisense oligonucleotides into primary osteoclasts can be used to study genes involved in osteoclast differentiation and activity.

Example 24: Capillary gel electrophoretic analysis of antisense oligonucleotide concentrations in bone compartments

[0209] The regulation through antisense mechanisms of genes involved in bone metabolism necessitates that antisense oligonucleotide be able to enter bone cells. Trabecular bone is the primary site of bone remodeling, thus it is especially important that oligonucleotides have access to the cells in trabecular bone. Chimeric oligonucleotides, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings", which consist of 2'-methoxyethyl (2'-MOE)nucleotides, can be visualized in

osteoblasts, osteoclasts, osteocytes and chondrocytes. It is also of importance to demonstrate that the oligonucleotides that do reach bone cells are intact, full-length oligonucleotides. In accordance with the present invention, the accessibility of oligonucleotide to bone compartments in vivo was assessed by measuring the concentration and size of oligonucleotide in different bone compartments.

[0210] Female Swiss-Webster mice (5–8 wks old) were fed a low-calcium diet and received daily subcutaneous injections of 10, 20, 30 or 50 mg/kg of ISIS 181071 or saline for two weeks. After the two week treatment period, mice were sacrificed and the cortical bone, bone marrow and trabecular bone compartments were isolated. Concentrations of full-length ISIS 181071 in each isolated bone compartment were measured by capillary gel electrophoresis. The results are expressed as micrograms of ISIS 181071 per gram of bone tissue. The data are shown in Table 10 and illustrate that the oligonucleotide of the present invention can be delivered to the cortical bone, the bone marrow and importantly to the trabecular bone in a dose-dependent manner.

Table 10

Capillary gel electrophoretic analysis of antisense oligonucleotide concentrations in bone compartments

	Concentration of full-length oligonucleotide, ug/g		
Dose of ISIS 181071	Cortical Bone	Bone Marrow	Trabecular Bone
10 mg/kg	13	22	35
20 mg/kg	39	36	71
30 mg/kg	32	64	138
40 mg/kg	31	49	152

Example 25: Short-term bone resorption model: time course of serum calcium levels after PTH infusion

[0211] Parathyroidectomized rats are a well-established, short-term model of bone resorption and are useful in the investigation of antiresorptive agents. However, it is difficult to surgically remove only the parathyroid gland of rodents, and the inadvertant removal of the adjacent thyroid gland often has effects on thyroid hormone levels that introduce into the experiment and undesirable level of variability. Continuous infusion of parathyroid hormone (PTH) into intact, young (6-9 weeks old) mice reproduces the effects seen in parathyroidectomized rodents and is thus a useful model for the study of antiresorptive agents. The PTH infusion activates bone osteoclasts and causes them to degrade bone matrix, with a resulting rise in serum calcium.

[0212] In accordance with the present invention, serum calcium concentration was evaluated in a short-term mouse model of bone resorption. Female, Swiss-Webster mice (5-8 weeks old) were placed on a low calcium diet and implanted with mini-pumps delivering PTH continuously. The pumps were calibrated to deliver 1ug PTH per 100g bodyweight per 6 hour time period (1ug/100g/6hr). With this procedure, over a 24 hour period, a total of 4ug PTH per 100g body weight is delivered (4ug/100g/24hr). PTH infusion was conducted in these mice for 6, 12, 18 and 24 hours. At the end of each time period, the mice were sacrificed and measurements were made of the serum calcium using the Sigma Diagnostics Calcium Kit (Sigma-Aldrich). Serum calcium is expressed as the percent increase relative to no PTH treatment. The results, shown in Table 11, are

the average of three mice per time point and demonstrate that PTH infusion increases serum calcium concentration as much as 230% after 24 hours.

Table 11

Serum calcium concentration in mice infused with PTH: time course study

Time	Serum Calcium Concentration
Hours	% control
0	100
6	109
12	115
18	125
24	230

Example 26: Serum calcium concentration in mice treated with RANK antisense oligonucleotide: dose response and delivery method comparison

[0213] In accordance with the present invention, serum calcium concentration was measured in mice treated with ISIS 181071 at different doses, delivered by subcutanteous mini-pumps or subcutaneous injection. Calcitonin, a known bone antiresorptive agent, was used as a control for antiresorptive activity.

[0214] Female Swiss-Webster mice (5–8 wks old), fed a low-calcium diet were treated with ISIS 181071 (SEQ ID NO: 34) for 15 days. The dose of oligonucleotide was either 30 mg/kg, 20 mg/kg, 10 mg/kg, 3 mg/kg, or 0.1 mg/kg administered by daily subcutaneous injections for the first five days, followed by mini-pump infusion for the remaining 10 days, or the dose was 10mg/kg or 30 mg/kg administered daily by subcutaneous injection for the entire 15 day period. No PTH was administered to the

mice that received oligonucleotide. A group of control mice were treated with either saline, PTH (1ug/100g/6hours) alone or calcitonin (400ng) plus PTH (1ug/100g/6hr). After 16 hours of PTH infusion (total PTH delivery of 3ug/100g) blood samples were taken from the tails of the mice and the serum calcium concentration was measured with the Sigma Diagnostics Calcium Kit (Sigma-Aldrich). The data are summarized in Table 12 and demonstrate that, with no PTH dose, the serum calcium concentration is similar with all of oligonucleotide doses and delivery methods tested.

Table 12

Serum calcium concentration in mice treated with RANK antisense oligonucleotide: dose response and delivery method comparison

	Serum calcium concentration (mg/dL)
saline	9
PTH	9
PTH + Calcitonin	8
ISIS 181071 injection and infusion	
30 mg/kg	9
20 mg/kg	9
10 mg/kg	9
3 mg/kg	9
0.1 mg/kg	9
ISIS 181071 injection	
30 mg/kg	8
10 mg/kg	9

Example 27: Serum calcium concentration and RANK mRNA expression in mouse proximal tibia after antisense oligonucleotide treatment and PTH infusion: dose response study and delivery method comparison

[0215] In accordance with the present invention, ISIS 181071 was evaluated for its ability to inhibit RANK expression and PTH-induced serum calcium concentration increase following 18 days of oligonucleotide treatment.

[0216] Female Swiss-Webster mice (5–8 wks old), fed a low-calcium diet, were treated for 18 days with ISIS 181071 (SEQ ID NO: 34) at different doses and through different delivery methods. The dose of oligonucleotide was either 30 mg/kg, 20 mg/kg, 10 mg/kg, 3 mg/kg, or 0.1 mg/kg administered by daily subcutaneous injections for the first five days, followed by mini-pump infusion for the remaining 13 days, or the dose was 10mg/kg or 30 mg/kg administered daily by subcutaneous injection for the entire 18 day period. After 18 days of treatment with oligonucleotides, the mice were infused with PTH by subcutaneously implanted mini-pumps at a dose of 1ug/100g/6hr. A group of control mice received saline, calcitonin (400 ng) plus PTH (1ug/100g/6hr) or PTH alone (1ug/100g/6hr). After 24 hours of PTH infusion (total PTH delivery of 4ug/100g), the mice were sacrificed and measurements were made of serum calcium with the Sigma Diagnostics Calcium Kit (Sigma-Aldrich) and RANK mRNA expression in proximal tibia (as described in other examples herein). The results are shown in Table 13 and are the average of four mice per group. Percent inhibition of RANK mRNA is normalized to PTH alone treatment. The data illustrate that the tested methods of oligonucleotide delivery can, in a dose-dependent manner, inhibit the expression of RANK mRNA and prevent the PTH-induced rise in serum calcium concentration

Table 13

Serum calcium concentration and RANK mRNA expression in proximal tibia after PTH infusion in mice: dose response study and delivery method comparison

	RANK mRNA %inhibition (normalized to PTH)	serum calcium (mg/dl)
Saline	47	11
PTH	0	20
PTH + Calcitonin	15	14
ISIS 181071 Dosed by injection and infusion		
30 mg/kg	32	17
20 mg/kg	33	18
10 mg/kg	23	18
3 mg/kg	5	20
0.1 mg/kg	19	21
ISIS 181071 Dosed by injection		
30 mg/kg	30	14
10 mg/kg	29	18

Example 28: Serum calcium concentration and RANK mRNA expression in mouse proximal tibia after antisense oligonucleotide treatment and PTH infusion: time course study

[0217] In accordance with the present invention, ISIS 181071 was administered to mice for different time periods to evaluate its ability to inhibit RANK expression and PTH-induced serum calcium concentration increase as a function of oligonucletide treatment time.

[0218] Female Swiss-Webster mice (5–8 wks old) were fed a low-calcium diet and received daily subcutaneous injections of 30 mg/kg of ISIS 181071 (SEQ ID NO: 34) for 2, 3, 5, 7, 10, 14, or 21 days. At the end of the oligonucleotide treatment period, mice were infused with PTH by subcutaneously implanted mini-pumps at a dose of 1ug/100g/6hr. A group of control mice were dosed with either saline, PTH alone

(1ug/100g/6hr) or calcitonin (400 ug) plus PTH (1ug/100g/6hr). Following 24 hours of PTH infusion (total PTH delivery of 4ug/100g), the mice were sacrificed and measurements were made of serum calcium with the Sigma Diagnostics Calcium Kit (Sigma-Aldrich) and RANK mRNA expression in the proximal tibia (as described in other examples herein). The data are the average of four mice per group and are summarized in Table 14. Percent inhibition of RANK mRNA is normalized to PTH alone treatment. The data demonstrate that treatment with the antisense oligonucleotide of the present invention lessens the PTH-induced increase in serum calcium concentration, and that this effect can be correlated with the inhibition of RANK mRNA in the proximal tibia.

Table 14

Serum calcium and RANK mRNA expression following antisense oligonucleotide and PTH infusion in mice: time course study

	Serum calcium concentration (mg/dL)	RANK mRNA % inhibition (normalized to PTH)	
		Proximal Tibia	Bone Marrow
Saline	11	59	8
PTH alone	21	0	0
PTH + Calcitonin	15	35	0
ISIS 181071 oligonucleotide treated			
21 days	15	52	51
14 days	15	53	52
10 days	18	37	35
7 days	16	61	39
5 days	16	32	47
3 days	17	14	30
2 days	20	34	0

Example 29: Serum calcium concentration after PTH infusion in mice treated with RANK antisense oligonucleotide: dosing schedule study

[0219] In accordance with the present invention, various antisense oligonucleotide dosing schedules were tested for their ability to inhibit serum calcium concentration increase following PTH infusion.

[0220] Female Swiss-Webster mice (5–8 wks old) were fed a low-calcium diet. The mice were dosed with the same total amount of ISIS 181071 (SEQ ID NO: 34) (450 mg/kg), however the frequency of the dosage was varied. The first group received daily injections for 2 weeks. The second group received daily injections of 30mg/kg/day for 5 days; the remaining 300mg/kg was divided into 12 injections over 24 days and were administered every other day. The third group received daily injections of 30mg/kg/day for 5 days; the remaining 300mg/kg was divided into 8 injections over 24 days and were administered every third day. The fourth group received daily injections of 30mg/kg/day for 5 days; the remaining 300mg/kg was divided into 6 injections over 24 days and were administered every fourth day. Each group contained eight mice. Treatment with oligonucleotides was followed with infusion of PTH by subcutaneously implanted mini-pumps at a dose of 1ug/100g/6hr. A control group received saline or PTH alone (1ug/100g/6hr). After 24 hours of PTH infusion (total PTH delivery of 4ug/100g), the mice were sacrificed and the serum calcium concentration was measured with the Sigma Diagnostics Calcium Kit (Sigma-Aldrich). The data are shown in Table 15 and are the average of eight mice per group. The data demonstrate that the various dosing schedules

of the oligonucleotide of the present invention can similarly prevent the PTH-induced rise in serum calcium concentration.

Table 15

Serum calcium concentration after PTH infusion in mice treated with RANK antisense oligonucleotide: dosing schedule study

	Serum calcium concentration (mg/dL)
Saline	9
PTH	21
injection schedule of ISIS 181071	
Daily	16
every other day	16
every third day	17
every fourth day	18

Example 30: Serum calcium concentration and antisense inhibition of RANK mRNA expression in proximal tibia after PTH infusion in mice: specificity and dose response

[0221] In accordance with the present invention, the specificity of antisense oligonucleotide inhibition of RANK was tested by comparing the effects of ISIS 181080 (SEQ ID NO: 43) to those of the 8-base mismatch ISIS 208565 (SEQ ID NO: 110).

[0222] Female Swiss-Webster mice (5–8 wks old) fed a low-calcium diet were treated for two weeks with either ISIS 181080 or ISIS 208565. The dose of oligonucleotide was either 40 mg/kg, 30 mg/kg, 20 mg/kg, or 10 mg/kg. After the 2-week treatment with oligonucleotides, the mice were infused with PTH by subcutaneously implanted mini-pumps at a dose of 1ug/100g/6hr. A group of control mice were dosed with either saline, PTH alone (1ug/100g/6hr) or calcitonin (400ng) plus PTH

(1ug/100g/6hr). After 24 hours of PTH infusion (total PTH delivery of 4ug/100g), the mice were sacrificed and measurements were made of serum calcium with the Sigma Diagnostics Calcium Kit (Sigma-Aldrich) and RANK mRNA expression in proximal tibia (as described in other examples herein). The results shown in Table 16 are the average from four mice per group. Percent inhibition of RANK mRNA is normalized to PTH alone treatment. The data demonstrate that the oligonucleotide of the present invention inhibits expression of RANK mRNA *in vivo* and consequently prevents the PTH-induced rise in serum calcium concentration. The data also illustrate that these effects are occuring due to an antisense mechanism.

Table 16

Serum calcium concentration and antisense inhibition of RANK mRNA expression in proximal tibia after PTH infusion: specificity and dose response

	RANK mRNA %inhibition (normalized to PTH)	serum calcium (mg/dl)
Saline	63	10
PTH	0	20
PTH + Calcitonin	2	15
ISIS 181080		
10 mg/kg	47	23
20 mg/kg	38	17
30 mg/kg	48	15
40 mg/kg	65	16
ISIS 208565		
10 mg/kg	0	21
20 mg/kg	0	17
30 mg/kg	19	18
40 mg/kg	7	16

[0223] As illustrated in this and other examples herein, the inhibition of PTH-induced serum calcium concentration by RANK antisense oligonucleotides (54% inhibition, n=4-8) is nearly as effective an inhibitor as calcitonin, a known antiresorptive agent (63% inhibition, n=4-8). If desired, additional antisense oligonucleotides can be screened in a like manner to identify those with more or less inhibitory properties.

Example 31: Antisense inhibition of RANK expression bone marrow: comparison in total bone marrow, monomyeloid and monocytic cell populations

[0224] Bone marrow consists of several different cell types. In particular, the monomyeloid and monocytic populations are known to express RANK. In accordance with the present invention, the inhibition of RANK mRNA expression by antisense oligonucleotides in subpopulations of bone marrow was evaluated.

[0225] Female Swiss-Webster mice (5–8wks old) fed a low-calcium diet were dosed daily with ISIS 181071 (SEQ ID NO: 34) at either 15 mg/kg or 30 mg/kg for 15 days in the absence of PTH. Following the dosing regimen, the mice were sacrificed and the bone marrow was collected and subdivided into monomyeloid (Gr-1 positive signal) and monocytic (Gr-1 negative signal) cell populations by fluorescence activated cell sorting. RANK mRNA expression was measured in total bone marrow, monomyeloid bone marrow, and monocytic bone marrow as described by other methods herein. The results are the average of at least three mice per group and are shown in Table 17. The data demonstrate that inhibition of RANK mRNA expression by the oligonucleotide of the

present invention occurs in a dose-dependent manner in the bone marrow cell types known to express RANK.

Table 17

Antisense inhibition of RANK expression bone marrow: total bone marrow, monomyeloid and monocytic cell populations

	RANK mRNA expression in bone marrow populations (% inhibition)		
Treatment	total bone marrow	monomyeloid	monocytic
Saline	0	0	0
ISIS 181071 15mg/kg	60	72	55
ISIS 181071 30 mg/kg	85	85	88

[0226] It is to be understood that the description, specific examples and data, while indicating exemplary embodiments, are given by way of illustration and are not intended to limit the various embodiments of the present disclosure. All references, GenBank accession numbers, information contained in a website, and the like, cited herein for any reason, are specifically and entirely incorporated by reference. Various changes and modifications within the present disclosure will become apparent to the skilled artisan from the description and data contained herein, and thus are considered part of the various embodiments of this disclosure.